University of Alberta

The Use of Milrinone in the Resuscitation of Asphyxiated Newborns

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Medical Sciences - Pediatrics

Edmonton, Alberta
Spring 2007

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Your file Votre référence
ISBN: 978-0-494-29969-2
Our file Notre référence
ISBN: 978-0-494-29969-2

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To Dennis,
for his encouragement, sense of humour and above all else, his patience.

Abstract

Neonatal asphyxia can lead to significant morbidity and mortality. After resuscitation, neonates can develop decreased cardiac function, pulmonary hypertension and multiorgan dysfunction. We hypothesized that milrinone, a phosphodiesterase type III inhibitor, could improve neonatal cardiopulmonary and regional hemodynamics after hypoxia-reoxygenation. Newborn piglets underwent hypoxia for two hours followed by reoxygenation and subsequent administration of placebo or one of three doses of milrinone in a blinded fashion. In a dose response manner, milrinone improved cardiac output, systemic oxygen delivery and decreased pulmonary vascular resistance without causing hypotension. Milrinone improved carotid and intestinal blood flow as well as oxygen delivery while decreasing regional vascular resistance at 0.75 μg/kg/min. There was no evidence of increased metabolic stress. We conclude that in a model of neonatal hypoxia-reoxygenation, milrinone is an effective inotrope for the improvement of systemic and regional hemodynamics without aggravating pulmonary hypertension, hypotension or metabolic dysfunction.

Acknowledgements

I would like to express my gratitude to my supervisor Dr. Po-Yin Cheung for his hard work, enthusiasm, and good nature. His encouragement and dedication has been infectious and provided avenues for further research.

I would like to thank Dr. David Bigam for his helpful shoves toward the finish line and his surgical expertise with the piglet model.

Thank you to Dr. Gary Lopaschuk for his guidance through the Master program and his knowledge in this research.

To my colleagues in our Neonatal Sciences Laboratory; Corinne Tymafichuk, Judi Li, Zakariya Al-Salam, Grace Chan, Marwan Emara, Aston Hugh, Raymond Lee, Mohammed Omar and Saapke Postma, John Ussher and Ken Strynadka of the Cardiovascular Research Group, I would like to thank them for their support and expertise.

I would like to acknowledge the contributions of the pathologists, Dr. Greg Charrois and Dr. Laurence Jewell for their analysis of the histological specimens, as well as Lynette Elder of Dr. Greg Korbutt's laboratory for the preparation of the samples.

For their financial contributions, I would like to gratefully acknowledge the Alberta Heritage Foundation for Medical Research, the Clinical Investigator Program of the Royal College of Physicians and Surgeons of Canada, the Canadian Institutes of Health Research, the Stollery Children's Hospital Foundation, the Department of Pediatrics and the Neonatal-Perinatal Medicine Fellowship Program.

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Abbreviations

ANOVA	analysis of variance
ATP	adenosine 3',5' triphosphate
BP	blood pressure
CAFI	carotid artery flow index
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
C_aO_2	arterial oxygen content
CI	cardiac index
C_vO_2	venous oxygen content
CVP	central venous pressure
FiO_2	fraction of inspired oxygen
GSSG	oxidized glutathione
H-R	hypoxia-reoxygenation
IV	intravenously
MAP	mean systemic arterial pressure
mm Hg	millimeters of mercury
OFR	oxygen free radical
P_aCO_2	arterial partial pressure of carbon dioxide
P_aO_2	arterial partial pressure of oxygen
PAP	mean pulmonary arterial pressure
PDE	phosphodiesterase
PHT	pulmonary hypertension
PVRI	pulmonary vascular resistance index
RAFI	renal artery index
$RADO_2$	renal artery oxygen delivery
RM	repeated measures
RAVRI	renal artery vascular resistance index
S_aO_2	arterial oxygen saturation
SMA	superior mesenteric artery
$SMADO_2$	superior mesenteric artery oxygen delivery
SMAFI	superior mesenteric artery flow index
SMAVRI	superior mesenteric artery vascular resistance index
SR	sarcoplasmic reticulum
SVRI	systemic vascular resistance index

CHAPTER 1

Asphyxia in the Neonate

ASPHYXIA

The word asphyxia is derived from the Greek "without pulse." Asphyxia is an event that causes reduced oxygen delivery and acidosis; usually leading to dysfunction of two or more organ systems. Asphyxia is a result of acute, chronic or acute on chronic episodes of severe hypoxemia. Hypoxemia is a state of reduced arterial oxygen blood content or arterial oxygen partial pressure.

The perinatal period is a time of complex and radical physiologic transitions for a neonate, from an intrauterine to extrauterine environment. While this transition often occurs without event, 10% of babies require some assistance at birth and 1% need extensive resuscitation.[1] There are neonates who require active resuscitation as consequence of perinatal asphyxia. Despite many advances in perinatology, perinatal asphyxia still occurs in 3/1000 births accounting for 19% of the 5 million neonatal deaths that occur each year.[2] Many more children and their families deal with significant morbidities resulting from asphyxia, such as cerebral palsy, learning disabilities, short gut syndrome and renal sequelae.

Despite the relatively common manifestations of asphyxia in the clinical realm of neonatology, the definition on perinatal asphyxia is still not absolute. The National Health and Medical Research Council report of the Health Care Committee Expert Panel on Perinatal Morbidity defined "perinatal asphyxia" as a condition in the neonate where there is the following combination:[3]

- An event or condition during the perinatal period that is likely to severely reduce oxygen delivery and lead to acidosis; and

- A failure of function of at least two organs (may include kidneys, heart, lungs, brain, liver and hematological) consistent with the effects of acute asphyxia.

The American Academy of Pediatrics and American College of Obstetricians and Gynecologists state that the term asphyxia should not be used applied to a neonate unless all of the following conditions are met:[4]

- Umbilical cord arterial pH < 7.0
- Apgar score of 0-3 at 5 minutes
- Neurologic manifestations (seizure, coma, hypotonia)
- Multisystemic organ dysfunction

These criteria are also used to define an intrapartum episode of asphyxia that may contribute to brain injury.[5] In Canada, the same criteria are used but also include the criteria of delayed onset of respiration for five or more minutes (requiring assisted ventilation at birth).[6]

Asphyxia can occur in the prenatal, intrapartum or post partum period and can be caused by maternal, obstetrical, placental, or fetal factors that decrease oxygen or blood flow to the maternal-fetal or neonatal system (Table 1-1). There are three main mechanisms for asphyxia to occur *in utero*: a) lack of oxygen secondary to placental insufficiency, b) an interruption in the blood supply of the umbilical cord, or c) lack of blood to the placenta (usually maternal hypoperfusion). Asphyxia during intrapartum can result in neonatal asphyxia secondary to failure of pulmonary or circulatory adaptation at birth.[7]

Perinatal asphyxia is known to cause multisystem organ dysfunction. The organ systems commonly affected are the kidneys, cardiovascular, pulmonary, and brain.

Asphyxia can result in acute renal failure, myocardial dysfunction, hypotension, pulmonary hypertension and coma which can lead to significant mortality and long term morbidity including physical and mental disability (Table 1-2).

It is hoped that recognizing fetal asphyxia and treating its neonatal manifestations will decrease morbidity and mortality in this patient population. To be able to support the neonate suffering from the sequelae of asphyxia, one must understand the underlying pathophysiology of asphyxia, reoxygenation and the imposed treatments.

TABLE 1-1: COMMON ETIOLOGIES OF BIRTH ASPHYXIA[8,9]

ETIOLOGY	EXAMPLE
Intrauterine	
Placental Hypoxia	Uteroplacental insufficiency Placental abruption Prolapsed or avulsed cord Chorioamnionitis Velamentous insertion
Blood loss/ Anemia - shock	Vasa previa Placenta previa Fetomaternal hemorrhage Fetal hemolysis Erythroblastosis Twin – to – twin transfusion Hemorrhage (intracranial, subgaleal, intraabdominal) Hydrops (congenital heart disease, infection, chromosomal)
Maternal illness	Maternal hypotension/ shock Eclampsia Maternal diabetes Maternal smoking/substance abuse Premature membrane rupture/ oligohydramnios
Fetal factors	Multiple gestation Chromosomal abnormality/ congenital malformation Intrauterine asphyxia

Intrapartum	
Birth trauma	Cephalopelvic disproportion/ large for gestation Shoulder dystocia Instrumented third stage (forceps/vacuum)
Uteroplacental unit	Umbilical cord compression Uterine tetany
Postpartum	
Central Nervous System	Maternal medications Trauma Chronic fetal hypoxia Chromosomal abnormalities
Neuromuscular Disorder	Myopathies
Infection	Maternal transmission (bacterial, viral) Iatrogenic
Pulmonary	Meconium aspiration Pulmonary hypoplasia Diaphragmatic hernia Respiratory distress syndrome Pneumothorax Upper airway malformation
Renal	Pulmonary hypoplasia/ Oligohydramnios

TABLE 1-2: MULTISYSTEM OUTCOMES OF NEONATAL ASPHYXIA

ORGAN SYSTEM	OUTCOME
Central nervous system	Hypoxic- ischemic encephalopathy Intracranial hemorrhage Periventricular leukomalacia Seizures Hypotonia/hypertonia Cerebral Palsy Developmental delay Death
Cardiovascular	Transient myocardial ischemia (cardiac stunning) Tricuspid valve regurgitation Poor myocardial contractility Bradycardia and dysrhythmias Hypotension
Pulmonary	Pulmonary hypertension and pulmonary hemorrhage
Renal	Acute tubular necrosis and acute renal failure
Adrenal	Adrenal hemorrhage and adrenal insufficiency
Gastrointestinal	Necrotizing enterocolitis and intestinal perforation Poor feeding tolerance Hepatitis and cholestasis
Metabolic	Inappropriate antidiuretic hormone secretion Hypoglycemia, hypocalcemia, hyponatremia Lactic Acidosis
Skin	Subcutaneous fat necrosis
Hematology	Disseminated intravascular coagulation Thrombocytopenia

THE EFFECTS OF HYPOXIA ON THE FETAL CARDIOVASCULAR SYSTEM

Despite many advances in perinatology, birth asphyxia results in over one million deaths world wide per year, and causes significant morbidity such as cerebral palsy, learning disabilities, and necrotizing enterocolitis. [10] Asphyxia is due to acute or chronic episodes of hypoxemia or a reduced arterial oxygen blood content (Table 1-3). [11]

Although the fetus is designed to develop in a relatively low oxygen environment, severe hypoxia can occur from decreases in maternal oxygen supply or uterine and umbilical blood flow.[12] During hypoxia, the fetus ensures its survival through cardiovascular alterations to ensure vital organ perfusion to the heart, brain and adrenals often at the expense of other body systems. Changes in blood flow can differ depending on the timing of hypoxia during fetal development, the duration and severity of hypoxia, the mechanism of the insult, and the fetal species being examined. Although much is known about the complex cardiovascular alterations resulting from chemoreceptors, sympathetic responses and catecholamines, further research into new novel local mediators including nitric oxide and adenosine are providing new questions to be answered in the fetal hypoxia literature.

TABLE 1-3: DIFFERENCES IN THE FETAL RESPONSE TO ACUTE OR CHRONIC HYPOXIA

	Acute Hypoxia	Asphyxia	Chronic Hypoxia > 48 h	Chronic Hypoxia > 1 week
Heart Rate	↓ then ↑	↓or ↑ then↓	↔	↔
Blood Pressure	↑	↑ then ↓	↔	↔
Movements	↓	↓	↔	↔
Cerebral Blood Flow	↑	↑ then ↓	↑	↑ from baseline ; ↓ from 48h hypoxia

Cardiovascular Response to Hypoxia

During acute hypoxia, the fetal cardiovascular response includes bradycardia resulting in increased arterial blood pressure and increased peripheral vasoconstriction. There is redistribution of combined ventricular output with increased blood supply to vasodilated arteries of the heart, brain and adrenals at the expense of blood flow to the kidneys, gut, muscle and skin. Prolonged episodes of hypoxia sustained by the developing fetus can lead to intrauterine growth retardation.

The initial decrease of oxygen in the fetal arterial blood will trigger a chemoreceptor response in the carotid bodies to increase their afferent response. This triggers a vagally mediated bradycardia. Much debate exists in the literature as to whether baroreceptors or chemoreceptors are responsible for the initial fetal hypoxia induced bradycardia. Blanco *et al* made direct afferent fiber recordings to show that carotid chemoreceptors were functionally active in the last trimester of fetal lamb gestation.[13] They found a high basal discharge in carotid receptors of fetal lambs that increased with decreasing PO_2.[14] However, these experiments were done under anesthesia which also increased catecholamines and PCO_2, making it difficult to determine just how each factor played into the increased discharge of carotid receptors. The bradycardia could be negated by bilateral carotid and aortic denervation in chronically instrumented sheep implicating the chemoreceptors.[15] Such denervation can also interrupt the baroreceptor response. It is also difficult to remove all afferent nerves through this process as aortic chemoreceptor afferents can be found in the vagus, and aortic afferents could have been confused with the cervical sympathetic trunk. Finally, selective chemodenervation showed that the bradycardia was caused by the carotid, not aortic, chemoreceptors.[16]

Incremental reduction of umbilical flow in a fetal sheep model demonstrated a linear response between fetal arterial carotid blood oxygen and fetal bradycardia. This occurred prior to changes in arterial blood pressure, PCO_2, or pH; thus, indicating a role for chemoreceptors response to PO_2 more than baroreceptors.[15] This experiment used incremental changes in umbilical flow. It does not address the marked changes in blood pressure and volume from umbilical occlusion that could also trigger baroreceptors for an additive effect on heart rate.

The vagally mediated bradycardia induced by hypoxia can be partially obliterated by atropine.[17] Lack of complete inhibition may indicate a direct hypoxic myocardial depression or stunning.

Following bradycardia, there is a sympathetically mediated vasoconstriction in peripheral vasculature, which can be abolished by fetal treatment with α-adrenergic receptor antagonists.[16] Studies of spinal cord transaction in sheep have demonstrated that systemic circulatory effects, such as increased arterial pressure, depend on sympathetic efferent activity and are independent of vagally mediated bradycardia.[13]

The α-adrenergic response produces a reflex vasoconstriction of skin, musculoskeletal, kidney and gut arteries, allowing a redistribution of blood and substrate flow to the heart, adrenal and brain. With continued hypoxia, endocrine pathways are recruited, increasing plasma concentrations of catecholamines, vasopressin, cortisol, angiotensin II, and neuropeptide Y in the fetus.[18-22] Each of these humoral factors maintains peripheral vasoconstriction and, together with local vasodilation in the brain, heart, adrenal glands, and umbilical cord allows redistribution of blood flow in the fetus during hypoxia.[23-25]

Catecholamines

With increasing duration and severity of hypoxemia, catecholamines levels increase 50-100 fold to support the sympathetic peripheral vasoconstriction and oppose vagally mediated bradycardia.[26]

The addition of hexamethonium to block neural input to the adrenals only mildly decreases the catecholamine levels in a young fetus. It abolishes catecholamine release in a late gestation fetus, implicating a neurally mediated release of catecholamines primarily from the fetal adrenal gland, as adrenal demedullation completely abolishes the rise in plasma adrenaline concentration and reduces the noradrenaline response to 10% of normal.[26,27] The remaining 10% of catecholamines is thought to be a spillover from stimulated sympathetic terminal nerve endings.

Fetal sheep manifest bradycardia and *increased* blood pressure with hypoxia. In contrast, chicken embryos have cholinergic regulation of their vasculature during hypotension.[28] Although chicken embryos and fetal sheep display many similarities, these differences illustrate the difficulties of working with animals models that may not reflect processes occurring in human fetal hypoxia.

Adrenocorticotropin Hormone, Cortisol, Vasopressin, and Renin-Angiotensin II system

Adrenocorticotropin hormone and cortisol increase during fetal hypoxia. Cortisol, but not adrenocorticotropin hormone release, decreases after carotid sinus removal or splanchic denervation, indicating a neural release pathway for cortisol.[29]

Vasopressin increases 50 fold during fetal hypoxia and results in bradycardia and hypertension. Vasopressin is detected early in gestation with local affects on the heart and

independent from chemoreceptor responses.[19] Exogenous vasopressin can produce the same blood redistribution effects as those during fetal hypoxia with redistribution of fetal blood flow away from peripheral organs. Measuring gestational vasopressin concentrations in response to hypoxia from decreased cord or placental blood flow, or supplementing a hypoxic fetus with exogenous vasopressin may elucidate its role in the distressed fetus.

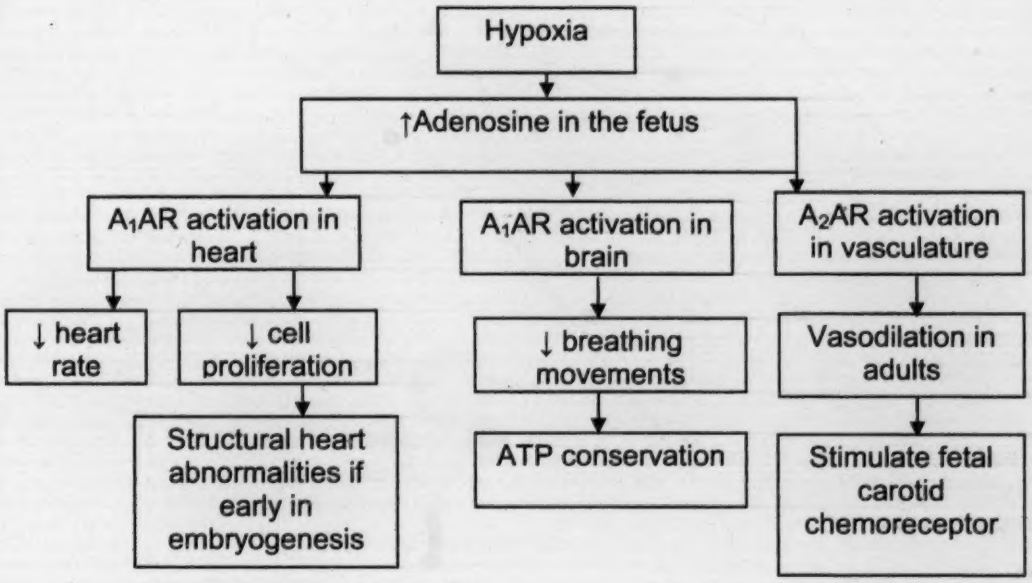
The renin-angiotensin system is present at 0.6 gestational age fetal sheep and can respond to small changes in blood volume. Administration of exogenous angiotensin II and inhibition of the endogenous angiotensin system by saralasin has demonstrated its ability to produce vasoconstriction in the fetus.[30]

Adenosine (Figure 1-1)

During fetal hypoxia, adenosine, a breakdown product of adenosine 3',5' triphosphate, is increasingly released from cells. It does not require synapses or a mature nervous system to function.[31] Adenosine A_1 receptors are expressed in embryonic fetal heart and brain and when activated will inhibit cardiomyocyte proliferation. Studies with adenosine reuptake blockers and adenosine A_1 receptor antagonists have demonstrated that adenosine can cause bradycardia and even asystole during early cardiac embryogenesis.[31] A body of research suggests a nonselective adenosine receptor antagonist prevents hypoxia induced fetal bradycardia and decreases femoral vasoconstriction in late gestation lambs hypothesizing that adenosine may stimulate the carotid chemoreceptor.[32] There are multiple types of adenosine receptors throughout the body, including in the carotid body. The use of a nonspecific receptor antagonist does not

specifically elucidate the adenosine pathways being blocked; thus, the conclusions of the authors that adenosine mediates the hypoxic carotid chemoreceptor response may not be valid. Interestingly, the adenosine receptor antagonist also attenuated the vasoconstriction of vasopressin and catecholamines, which are chemoreceptor independent pathways.

FIGURE 1-1: POSSIBLE ROLES OF ADENOSINE IN FETAL HYPOXIA



A_1AR : Adenosine A_1 receptor

A_2AR : Adenosine A_2 receptor

ATP : Adenosine 3',5'- triphosphate

Blood Flow Distribution and Cardiac Workload for Differing Etiologies of Hypoxia

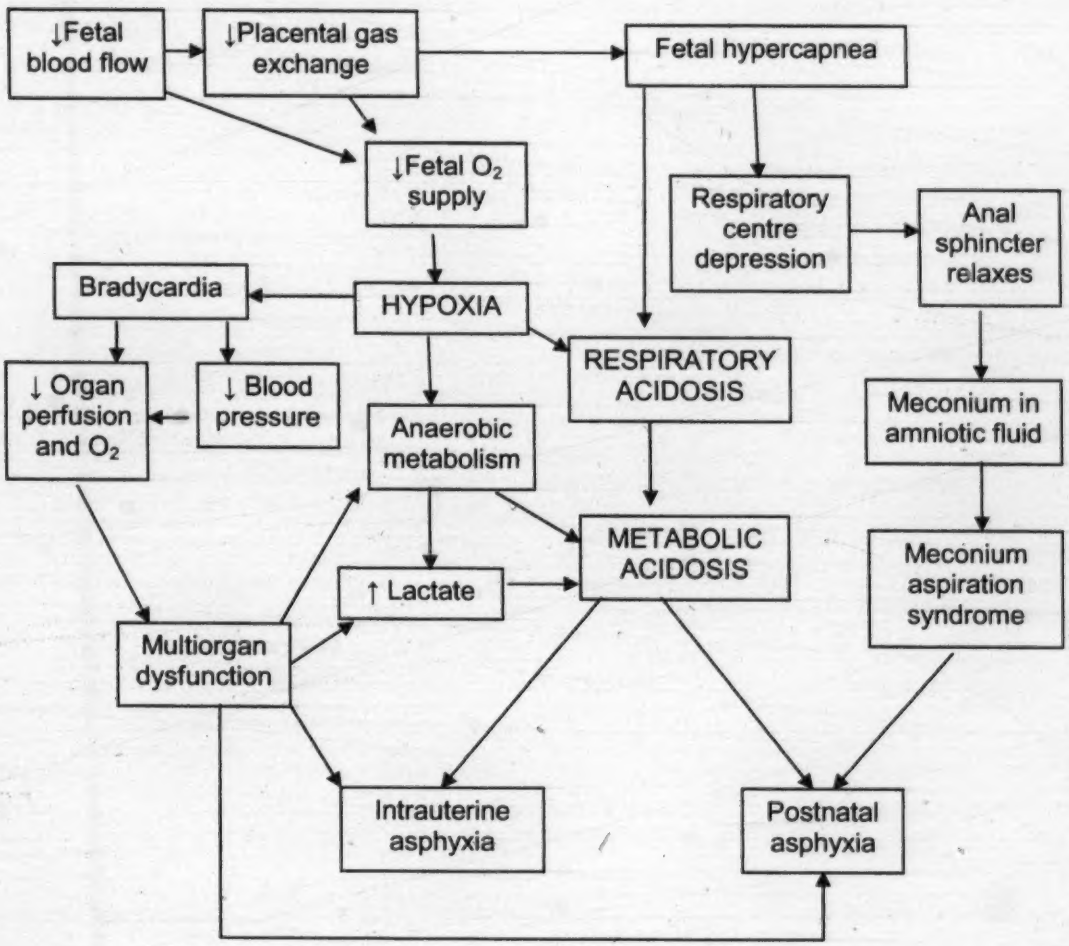
Using radionucleotide labeled microspheres in fetal animal models, researchers have examined how hypoxia affects blood flow to individual fetal organs.

Using hypoxic ewes, Rudolph *et al* found that combined ventricular output fell by 20% in fetal lambs of 0.8 gestation with mild acidemia and was associated with a 40% drop in blood flow to the fetal body.[33] The umbilical-placental blood flow did not change significantly. Compared to normoxic control fetuses, hypoxia increased blood flow to the myocardium (250%), the brain (180%), and the adrenal glands (250%). Blood flow to the kidney and gut fell to 50%, musculoskeletal system to 30%, with negligible lung flow.

Maternal hypoxia decreased the oxygen content of the blood delivered to the fetus through decreased venous oxygen content. Clinically, maternal hypoxia tends to be a more chronic insult, often occurring throughout the pregnancy in the form of high altitude living or smoking. This clinical aspect is not reflected in many of the initial benchmark experimental models in the literature. Further studies determined that mechanisms of hypoxia from reduced blood flow, such as uterine or umbilical blood flow reduction, had different redistributions of blood flow than those found in the maternal hypoxia model which maintains flows but decreases venous oxygen content.[33] Blood flow still increases to the brain, adrenals and heart but peripheral organ flows do not change significantly except for the lungs. Blood flow across the ductus venosus increases but to a lesser extent than the increase in venous return from the inferior and superior vena cava. With severe arrest of uterine or umbilical flow, hypoxia worsens, PCO_2 climbs, pH drops and asphyxia can result (Figure 1-2). In a fetal sheep model of total cord occlusion, the *initial* vasoreactive mechanisms and blood distribution were found to be the same as for milder

decreases in umbilical flow, but continued asphyxia produced profound differences.[34] The heart rate became tachycardic or bradycardic, and within 3 minutes of asphyxia, cerebral blood flow ceased to increase and the fetus directed blood to the brainstem at the expense of the cerebrum to preserve vital systems like the breathing centre. Within an additional minute of asphyxia, the peripheral resistance fell, blood pressure decreased, central organ resistance increased with reduced adrenal blood flow. Asphyxia results in severe heart failure and increased preload that translates into an increased umbilical venous resistance making further placental-umbilical blood flow even more difficult.

FIGURE 1-2: PATHOPHYSIOLOGY OF ASPHYXIA[7]



The fetus relies on umbilical venous blood flow to supply oxygen and nutrients. During fetal hypoxia, ductus venosus tone is altered via adrenergic innervation, nitric oxide and prostaglandins allowing an increase of 10-15% in umbilical venous blood to flow through the ductus venosus bypassing the liver to increase oxygen delivery to the fetus.[33] This increase in ductus venosus blood flow occurs with all mechanisms of hypoxia. Using microspheres, it was found that blood originating in the ductus venosus preferentially streams into the right atrium, through the foramen ovale, into the left atrium and ventricle to be expelled through the aortic root to perfuse the coronary arteries and the brain with oxygenated blood.[30] With increasing peripheral vasoconstriction and cerebral vasodilation, changes in cardiac afterload occur. The fetal heart has limited ability to increase stroke volume in response to increased afterload, and compensates for reduced cardiac output by increasing fetal heart rate. In asphyxia, the hypoxia induced vagal bradycardia can be difficult to overcome and reduced cardiac output ensues. Cytochrome c, a mitochondrial complex, is found to be higher in hypoxic fetal hearts, possibly indicating a metabolic adaptation to higher workload.[35] When the heart begins to fail due to hypoxic myocardial dysfunction and increased afterload, an increased end diastolic pressure gradient develops between the right atrium and the ventricle. Umbilical venous blood flow into the heart becomes increasingly difficult resulting in the reversal of ductus venosus flow, or in serious cases of fetal distress, umbilical vein flow reversal.

This knowledge of fetal physiology, garnered in the lab with chronically instrumental fetal sheep, has had valuable translational value in the medical field of perinatology. Using Doppler ultrasound, perinatologists can use middle cerebral arteries and umbilical vessel flows to determine if a fetus is in distress. Hypoxic vasoconstriction

can reverse or negate umbilical arterial flows. With hypoxia, cerebral artery resistance is decreased due to vasodilation. A sudden loss of cerebral vasodilation precedes fetal death by hours and requires intervention.[36] Doppler studies following "at risk" fetuses over time, may find the trend of changes in Doppler correlate more with biochemical markers of fetal distress at delivery. Using this approach, Doppler ultrasound has reduced perinatal deaths in pregnancies associated with uteroplacental insufficiency.[37] Being able to monitor the fetus and its response to hypoxia may allow delivery at appropriate times and minimize difficulties that an asphyxiated neonate may have in adapting to extrauterine life.

CONCLUSION

Despite advances in perinatal and obstetrical medicine, severe perinatal asphyxia and the associated morbidities still occur. These morbidities become the responsibility of the neonatal intensive care and often take the form of organ dysfunction, hypotension, pulmonary hypertension and low blood flow with poor oxygenation. Perinatal asphyxia is known to cause multisystem organ dysfunction which leads to significant mortality and long term morbidity including physical and mental disability.

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CHAPTER 2

Consequences of Asphyxia on Organ Systems

MULTIORGAN DYSFUNCTION

Multiorgan dysfunction is a common clinical feature of neonates who have sustained a significant asphyxial insult. It is thought to be secondary to the redistribution of blood flow away from non-vital organs that occur during fetal and neonatal asphyxia, resulting in dysfunction in one or more non-essential organs. This dysfunction must be monitored and treated in a neonatal intensive care unit. The organ systems commonly affected are the kidneys, liver, cardiovascular, pulmonary, and brain. Acute renal failure and tubular necrosis may occur in the kidneys. In the gastrointestinal system, the bowel may sustain ischemia and necrosis while the liver may be unable to synthesize proteins and utilize enzymes properly leading to a coagulopathy or temporary liver failure. The cardiovascular system may be affected by myocardial stunning or dysfunction and hypotension while the lungs can manifest pulmonary hypertension. Neurologically affected neonates can range from subtle changes in muscle tone to obtunded.

In a study of 130 asphyxiated neonates with neurologic involvement, 86% had respiratory involvement requiring mechanical ventilation, 85% had hepatic dysfunction (increased aspartate aminotransferase or alanine aminotransferase within 1 week of event), 70% had renal involvement (oliguria with increased serum creatinine), and 62% with cardiovascular dysfunction (hypotension requiring inotropes at < 24h of age or an electrocardiogram consistent with myocardial ischemia).[1] This study used very broad definitions of respiratory involvement and it can be argued that much of the respiratory involvement was actually secondary to neurologic dysfunction causing apnea. Martin-Ancel *et al* evaluated a series of 72 asphyxiated term neonates and found 82% of them had one or more organ systems involved.[2] Severe central nervous system injury was

always associated with multiorgan dysfunction syndrome. Renal, pulmonary, cardiac and gastrointestinal involvement was found in 42%, 26%, 29% and 29%, respectively. No association of the severity of organ dysfunction was found with umbilical cord arterial pH or meconium stained fluid. In 35 asphyxiated term neonates, Perlman *et al* demonstrated an asphyxial involvement of the kidneys (40%), central nervous system (31%), cardiovascular (36%), pulmonary (23%) and gastrointestinal system (rarely).[3]

Neonates with serious neurologic involvement often have a poor with prognosis with 55-65% suffering from adverse outcomes such as death or major neurological sequelae.[1,4] As the number of organs involved increases from one to three, the rates of adverse outcomes increases.[1]

Differences in the prevalence of organ involvement between studies may be related to different and arbitrary criteria for organ dysfunction. Regardless, many asphyxiated infants require intensive care and suffer from long term morbidity secondary to the organ dysfunction caused by neonatal asphyxia.

DIFFERENCES IN THE FETAL, NEONATAL, AND ADULT HEART

Cardiac Programming in Early Embryogenesis

Apoptosis is important for morphogenesis, including that of the heart. Any alteration in apoptosis or cardiomyocyte development could potentially affect cardiac function for life. Cardiomyocytes are highly specialized cells that only replicate for a few months after birth. Chick embryos incubated into late gestation under mild hypoxic conditions were found to have cardiovascular structural and functional abnormalities including aortic hypertrophic growth, left ventricular dysfunction and sympathetic hyperinnervation in the form of increased vascular resistance and reactivity of peripheral arteries (mesenteric) that preexisted birth.[5]

Using nucleic acid array technology, the expression of 48 of the 498 genes examined in hypoxic fetal rat hearts had been changed by hypoxia.[6] Upregulated genes included those needed during hypoxic stress, while genes for proliferation and survival were downregulated. While the expression of many genes is altered, studies still need to be done as to how their interactions are altered. In a hypoxic fetus, the hypoxia-inducible factor pathway is upregulated.[7] Endothelin-1 and vascular endothelial growth factor are downstream target molecules for hypoxia-inducible factor and known to be involved in vascular smooth muscle and cardiac hypertrophy.

Neonatal and Adult Hearts

The heart is regulated by a predictable series of modifiable events. Influx of intracellular calcium causes the contraction of elements of the sarcomere in heart muscle during systole. Decrease in calcium availability causes muscle relaxation, or diastole.

The contractile elements of the sarcomere consist of the thin filament, made of α-actin, and the thick filament, made of myosin. Myosin is made of two heavy chains, the α or β heavy chain. The myosin head binds actin. With the hydrolysis of adenosine 3',5' triphosphate (ATP), the myosin head pivots causing movement of the actin filament, shortening of the sarcomere and contraction of the muscle. Myosin and actin binding is calcium dependent via the troponin complex: Troponin I, C and T.

In the neonatal ventricle, most of the actin is cardiac α-actin, while the adult heart has a greater component of skeletal α-actin. Neonatal myosin is predominantly the V3 isoform consisting of two β heavy chains. The neonatal heart contains mostly the skeletal isoform of Troponin I while adult hearts change over to the cardiac isoform.[8]

As the binding and release of actin and myosin is calcium dependent, maturational differences in calcium handling, intracellular versus extracellular calcium sources, and differences in calcium transport may explain some differences in the response of neonatal and adult hearts to asphyxia.

Inward movement of calcium into the cell is mediated by an action potential generated by a slow inward calcium current from an L-type calcium channel. This channel is voltage gated. Once a level of depolarization occurs, the channel opens and calcium moves intracellularly. Increase in intracellular calcium causes further release of intracellular calcium stores from the sarcoplasmic reticulum (SR).

The SR in neonates is relatively underdeveloped compared to that found in the adult myocardium.[8] In neonates, the initial movement of calcium through the L-type calcium channel is the main contributor to the calcium pool that binds the troponin complex to initiate cardiac contraction as opposed to the intracellular store from the SR

which is the main calcium contributor to contraction in the adult heart.[9] This difference may be important during asphyxia-reoxygenation in neonates, as reperfusion releases calpain I, a calcium dependent cysteine protease, that may inhibit L-type calcium channels. As neonates are particularly dependent on this form of calcium channel for ventricular contraction, poor calcium influx may result in myocardial stunning compared to adult myocardium that can rely on other forms of calcium handling.

Diastole, or muscle relaxation in the heart, is caused by the resequestration of calcium from the cytosol back into the SR or extracellular space. This is predominantly accomplished by the SR calcium ATPase with a minor contribution from another sodium-calcium exchanger. The phosphorylated phospholamban protein (by cAMP dependent protein kinase A) activates SR calcium ATPase. The contribution of the sodium-calcium exchanger to calcium removal during diastole in neonates may be increased due to the SR immaturity in neonatal hearts.[8]

Neonatal hearts have a higher basal contractile state with decreased compliance compared to older children.[10] Due to a decreased contractile reserve, the neonate must increase heart rate in response to stress or increased afterload. A decrease in neonatal myocardium compliance may cause increased end diastolic ventricular pressures during higher doses of inotropic support.

MYOCARDIAL DYSFUNCTION SECONDARY TO ASPHYXIA

When asphyxia occurs in the perinatal period, the neonate is programmed to preserve vital organs, mainly the heart and brain.[11] However, occasionally the compensatory mechanisms are not enough to prevent damage to the heart resulting in transient myocardial ischemia, also known as hypoxic myocardiopathy or myocardial stunning. Myocardial ischemia of the newborn was initially described in full term infants by Rowe and Hoffman in 1972.[12]

It is known that myocardium can sustain short intervals of myocardial hypoxia, and even ischemia, without cell death. As the severity of the ischemia progresses, the cardiomyocytes sustain greater damage and are at risk for greater reperfusion- associated injuries. Reperfusion of the coronary arteries is necessary after hypoxia to salvage the myocardium. However, this reperfusion may cause cardiomyocyte dysfunction such as myocardial stunning, microvascular and endothelial injury, and irreversible necrosis.[13]

Myocardial stunning was defined in 1975 by Heyndrickx *et al* when they found that reperfusion of ischemic myocardial tissue resulted in a period of prolonged, yet reversible, contractile dysfunction.[14] This finding led to the current definition of myocardial stunning as "prolonged post-ischemia dysfunction of the viable tissue salvaged by reperfusion."[15]

The hypothesized mediators of myocardial reperfusion injury and the subsequent myocardial stunning include oxygen free radicals (OFR), intracellular calcium overload, endothelial and microvascular dysfunction and altered myocardial metabolism.[13] When molecular oxygen is reintroduced to an ischemic myocardium, it can undergo a series of reductions to form OFR such as superoxide anion, hydroxyl radical and peroxynitrite

which contribute to reperfusion injury.[16] Enzymes within the myocardium including xanthine oxidase, cytochrome oxidase and cyclooxygenase can also produce OFR. The OFR can damage polyunsaturated fatty acids in the cell resulting in lipid peroxides and hydroperoxides which cause further damage to the sarcolemma and membrane bound enzymes.[13] Endothelial release of platelet activating factor and alterations of nitric oxide are also mediated through OFR.

Oxygen free radicals can act as a vasoconstrictor in the coronary vasculature. This exaggeration of endothelium dependent vasoconstriction, coupled with impairment of endothelium dependent vasodilation, further reduces blood flow to already dysfunctional myocytes.

Ischemia and reperfusion are linked to an increase in intracellular calcium and an altered myofilament sensitivity to calcium. L-type calcium channels. This process may be responsible for an increased influx of calcium into the sarcolemma.[17]

Hypoxia and the Neonatal Heart

The adaptations of a neonatal heart to hypoxia are multifaceted as are the underlying mechanisms that can lead to cardiac dysfunction. The newborn heart has a higher resting ventricular output per kilogram of body weight compared to the adult leading to a decreased reserve capacity and a limited response to hypoxia and increased preload.[18]

During short term hypoxia in neonatal lambs (fraction of inspired oxygen = 0.08-0.1), there are increases in heart rate, myocardial blood flow and myocardial oxygen consumption with no significant change in arterial blood pressure. There is no change in

left ventricular end diastolic pressure but significant increases in maximum dP/dt[i] and dV/dt[ii] but not as a function of increasing hypoxia. The increase in dP/dt and dV/dt during hypoxia indicates an increase in left ventricular contractile function. With the increase in left ventricular systolic pressure and increased contractile strength, increased myocardial workload causes myocardial oxygen consumption to increase. Inefficient metabolism follows, leading to myocardial depression.[19] With increasing severity of hypoxia, cardiac output begins to fail. In an isolated rabbit heart model, hearts were exposed to 90 minutes of 10% coronary blood flow and 30 min of reperfusion.[20] Compared to controls, hearts recovered only 50-70% of the peak systolic pressure. The tissue ATP and glycogen were both markedly reduced with accompanying decreased myocardial oxygen consumption. There was also a marked increase of ventricular wall stiffness. All of these factors would contribute to heart dysfunction after hypoxia-reoxygenation.

Myocardial stunning can also be a result of delayed return of normal cardiomyocyte metabolism. During prolonged or severe asphyxia, the fetus or neonate shifts from aerobic oxidation to anaerobic glycolysis with lactate production.

Proinflammatory cytokines, most commonly interleukin-6 and -8 and tumour necrosis factor alpha may also depress myocardial function post reperfusion. Although the mechanism is unknown, interleukin-6 is known to increase nitric oxide production and tumour necrosis factor alpha can alter calcium influx into the sarcomere.[21]

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i A measure of left ventricular force or the rate of left ventricular pressure increase during systole
ii Determinant of stroke volume during systole or the left ventricular ejection rate during systole

In a study by Tapia-Rombo *et al*, as much as 82.5% of severely asphyxiated term infants showed myocardial stunning on echocardiography and electrocardiogram of which 70% of the patients had cardiovascular symptoms.[22]

Left ventricular dysfunction, with occasional mitral regurgitation and ventricle wall motion abnormalities, is found with transient myocardial ischemia post asphyxia. Severe hypoxia results in a decrease in left ventricular systolic function as evidenced by a reduced shortening fraction[iii] and a depressed ejection fraction.[iv 23] Shortening and ejection fraction are load dependent and can be misleading during hypovolemia.[24]

Tricuspid insufficiency is often found post asphyxia as neonatal myocardial ischemia has a predilection for papillary muscles of the tricuspid valve, disallowing further adaptation of the tricuspid valve to changing hemodynamic pressure during further hypoxia or reperfusion. A large series of autopsies of asphyxiated neonates who had died within a week of life who had tricuspid or mitral regurgitation demonstrated a high incidence of necrosis of the papillary muscle.[25]

Wittnich *et al* compared term neonatal and adult porcine hearts ex vivo and determined that neonatal hearts were more susceptible to hypoxia and ischemia than adult hearts.[26] They determined the time between ischemia and the beginning of contracture. They found that the time to ischemic contracture was significantly shorter in neonatal hearts. The immature myocardium has a greater anaerobic glycolytic capacity. Research with rat neonatal myocardium has demonstrated that lactate concentrations were significantly higher for a given amount of ischemia when compared to adult hearts.[27] With prolonged asphyxia, there is a depletion of the sparse neonatal cardiac glycogen

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iii The fractional change in left ventricular short axis dimension from end-diastole to end-systole
iv The change in left ventricular volume from end-diastole to end-systole

reserves; thus, neonates undergo hypoglycemia and anaerobic metabolism with an accumulation of lactate. Increased lactate and acidosis increases pulmonary vascular pressures and vasoconstriction causing increased cardiac afterload and workload on an already dysfunctional heart. Even after hypoxia or ischemia has ceased, lactate release persists and mitochondrial pyruvate dehydrogenase activity remains depressed for up to 30 min after reperfusion.[27] Histological and biochemical studies in lambs after a 60 min asphyxial event demonstrated a persistence of lactate in the heart 72h later. Other organ systems had cleared the lactate by this time.[28] Increased lactate accumulation may result in a decrease in ventricular function even after the resolution of hypoxia as well as a greater tissue acidosis upon reperfusion.[29]

Early research into acidosis on myocardial function used isolated heart preparation of adult mongrel dogs. A drop in pH with a consequent rise in PCO_2 resulted in decreased left ventricular contractility. A rise in PCO_2 alone with normalized pH was not sufficient to alter ventricular function leading the authors to conclude that the change in ventricular contractility was due to an alteration in intracellular pH.[30] In support of this hypothesis, Fisher's in vivo lamb study found that acidemia was associated with decreased cardiac output, decreased dP/dt, and a reduced aortic mean blood pressure (BP).[31] With the decreased cardiac output, there was a reduction in coronary blood flow and oxygen consumption. This was associated with a decreased ability for the heart to relax during diastole resulting in an increased left ventricular end diastolic pressure and systemic vascular resistance. With the normalization of pH, cardiac output returned to normal but ventricular force was temporarily depressed providing another mechanism for cardiac stunning after asphyxia.

Further research in the same model compared the effects of acidemia and short term hypoxia (20 min) on neonatal cardiac output and regional blood flows.[32] Hypoxia increased cardiac output as well as cerebral and myocardial blood flows with decreased blood flow to the kidneys and little change in flow to the gastrointenstinal tract or carcass. Acidemia decreased cardiac output with subsequent decreased blood flow to the myocardium, brain, gastrointestinal tract and carcass. With prolonged hypoxia, acidemia prevails which may explain why initial increases in cardiac and cerebral flow with hypoxia eventually evolve into decreased flows.

Asphyxiated infants with myocardial dysfunction can present with peripheral and pulmonary vasoconstriction. Resultant pulmonary arterial hypertension can also reverse the direction of blood flow through a patent ductus arteriosus and foreman ovale aggravating cyanosis and hypoxia.[23] An increased cardiac workload from pulmonary hypertension can increase cardiac transmural pressure decreasing the perfusion in the subendocardial layer and causing further dysfunction.[22]

Myocardial dysfunction found post asphyxia can be associated with poor cardiac output and consequently, poor organ perfusion and decreased oxygen consumption. Clinically, this presents as hypoxemia, decreased urine output, hypotension, echocardiographic findings of decreased heart function and lactic acidosis.

During severe asphyxia and post reoxygenation, myocardial dysfunction persists with peripheral vasodilation.[24] A tailored approach to medical cardiovascular support is needed to ensure that medical interventions to increase blood pressure does not result in excessive vasoconstriction, increased cardiac workload or dysfunctional organ tissue oxygenation.[33]

THE CARDIOVASCULAR RESPONSE AND ORGAN PERFUSION WITH HYPOXIA

Organ Perfusion and Blood Pressure

Organ perfusion is dependent on net perfusion pressure across the organ and peripheral vascular resistance or simply stated: Blood flow = arterio-venous blood pressure difference ÷ vascular resistance.[34] If absolute venous pressure is low, arterial BP can be substituted for the pressure gradient. As arterial BP is easy and convenient to measure, clinicians have used BP to determine circulation. A recent review by Osborn in the hypotensive preterm population demonstrates a complete lack of support for treating a low BP in the hopes of preventing morbidity.[35] This paper did not address the hypotension of the asphyxiated term newborn.

In non-instrumented neonates, superior vena cava blood flow corrected for weight can be monitored clinically to estimate total systemic perfusion. Doppler velocities of large arteries supplying peripheral organs can also be measured. While Doppler ultrasound allows clinicians to compare perfusion between weights and gestations, it is also operator dependent allowing for variations in the literature. Mean arterial BP is poorly correlated to systemic perfusion as measured by superior vena cava flow or left ventricular output.[36,37]

If BP does not seem to correlate to organ perfusion and subsequent morbidity, decreased peripheral perfusion may be affected by an increased peripheral resistance. An elevated vascular resistance would compensate for the decreased cardiac output after asphyxia but may also impair peripheral perfusion.

Hypoxia and Blood Flow

When term fetal and postnatal animals undergo hypoxia, there is a rapid decrease in renal and mesenteric blood flow with increased vascular resistance. The speed at which the changes occur suggest a neural mechanism rather than a local response. Experimental models have provided evidence that stimulation of the alpha adrenergic sympathetic nervous system regulates renal and gut perfusion during asphyxia.[38] During severe hypoxia, the central nervous system redistributes blood flow to organs by differentially shunting blood away from peripheral organs and supporting blood flow to the heart and brain. This process is mediated by alpha adrenergic receptors.[39,40] With continued hypoxia, there is a failure of the fetal blood redistribution and vasoconstriction which corresponds to a drastic fall in BP.[41] Fetal adaptations to intrauterine hypoxia include prolonged secondary hypoperfusion with reduced metabolism. It is hypothesized that this may be due to the overwhelming acidosis causing vasoparesis or a change in the sympathetic activity or loss of effective response to catecholamines.[34] If this secondary hypoperfusion is present following a hypoxic insult, the neonate becomes vulnerable to a secondary insult. There is a limit to the fetal adaptive response to hypoxia at which point severe cardiovascular compromise occurs; thus, the fetal adaptation becomes deleterious to the neonate.[34]

Upon recovery from hypoxia, a persistent hypoperfusion of peripheral organs (intestine, kidney) has been demonstrated. This hypoperfusion is associated with continued increases in vascular resistance and an improved BP which may help support the continued diversion of blood to the recovering heart and brain.[41] This theory is supported by the work in fetal sheep demonstrating latent gut perfusion was restored post

hypoxia only when mesenteric vascular resistance decreased and not when BP increased.[34] This change in vascular resistance may be due to the eventual decrease in sympathetic tone as an infusion of an alpha adrenergic blocker post hypoxia alleviated latent gut hypoperfusion.[42] The role of prolonged hypoperfusion post asphyxia is still not understood. It is not known if this is a maladaptive response that may potentially lead to significant morbidity in the postnatal period.

Piglet Model of Cardiovascular Response and Organ Flow: Hypoxia and Reoxygenation

Neonatal swine models of hypoxemia and reoxygenation have demonstrated the multisystem effects of this affliction and its treatment. Cheung *et al* exposed acutely instrumented anaesthetized newborn piglets to 3h of hypoxia (FiO_2= 0.10-0.15, PaO_2 30-40 mmHg) followed by reoxygenation for 30 min at FiO_2=1.0.[43] These piglets were compared with normoxic controls. By 3h of hypoxia, all piglets developed a lactate acidosis. The cardiac index and stroke volume had increased over baseline to a maximum at 30 min of hypoxia but declined in the last hour of hypoxia back to a baseline level. Systemic BP decreased through hypoxia with a decrease in systemic vascular resistance. Pulmonary arterial pressure and vascular resistance both increased. Mesenteric blood flow decreased with an increase in mesenteric vascular resistance during the first 15 min of hypoxia but returned to baseline values for the remainder of hypoxia. Systemic oxygen delivery was decreased by 50% and mesenteric oxygen delivery fell to 30-40% of baseline. These decreases were associated with increased oxygen extraction in the systemic and mesenteric circulations.

There was a continued suppression of cardiac index, BP, and systemic delivery of oxygen below baseline despite resuscitation with 100% oxygen. Pulmonary arterial pressure partially fell towards normal with a decrease in pulmonary vascular resistance. Systemic oxygen extraction return to baseline. Mesenteric blood flow and oxygen delivery returned to normoxic baseline after 30 min of oxygen resuscitation.

This study confirmed that the degree and length of hypoxia alters the neonate's ability to respond and compensate for the stress of hypoxia. The inability of the animal to maintain compensatory mechanisms may have been related to the increased lactate levels, acidosis and ATP depletion that accompany prolonged asphyxia and are encountered clinically in the neonatal intensive care unit. This study also demonstrated both an initial hypoxic vasoconstrictive response of the mesenteric vasculature followed by a vasodilation with prolonged hypoxia. This study adopted a temporal but continuous approach to blood flow helped to account for some of the differences found in previous studies of varying hypoxia lengths and severity with respect to vasodilation versus vasoconstriction of the mesenteric vasculature upon hypoxic stresses.

HYPOXIA-REOXYGENATION AND OXYGEN FREE RADICALS

Reoxygenation of a hypoxic neonate particularly with high concentrations of oxygen, releases excessive amounts of OFR and inflammatory mediators which can lead to cell dysfunction and death.[44] A surge of nitric oxide and superoxide anion production occurs upon reoxygenation. Neonates may be at risk for OFR injury, especially those with prematurity and perinatal asphyxia because their anti-oxidant system is compromised. Oxygen free radicals lead to lipid peroxidation and deplete reducing molecules such as nicotinamide adenine dinucleotide phosphate and glutathione, as well as activate matrix metalloproteinases.[45] Oxygen free radicals can inflict damage upon reoxygenation in many different organ systems.

HYPOXIA- REOXYGENATION AND THE BRAIN

Hypoxia-ischemia

Encephalopathy secondary to asphyxia can lead to death or major disability. Peliowski and Finer reviewed 5 studies of term newborns with hypoxic ischemic encephalopathy and determined that the risk of death or disability was 5.6% and 20% respectively in moderate encephalopathy and 60% and 72% in severe cases.[46]

Autoregulation of cerebral blood flow is often impaired in neonates who have sustained hypoxia or asphyxia.[47] Debate exists as to the contribution of impaired autoregulation as a cause of hypoxic ischemic encephalopathy in the asphyxiated neonate.

Autoregulation is an artery's ability to constrict or relax in response to increases or decreases in transmural pressure, respectively. Autoregulation allows organs to maintain a constant blood flow during variations in blood pressure or vascular resistance. Loss of cerebral autoregulation leads to a pressure-passive circulation where low BP results in low cerebral blood flow.[48]

Blood flow to the brain is the result of perfusion pressure, defined as the pressure gradient between arterial-venous pressure and intracranial pressure. Blood flow is also controlled by vascular resistance caused by variable tone of smooth muscle cells altering the diameter of blood vessels and blood viscosity. Factors hypothesized to alter cerebral vascular tone include BP, PCO_2, PO_2, cellular metabolism and neurogenic. In the cerebral vasculature, hypocapnia causes vasoconstriction while hypercapnia can cause vasodilation.

Hypoxia is a cerebral vasodilator via hyperpolarization of smooth muscle cells. With continued hypoxia, the vasodilatory response becomes maximally activated such that any superimposed hypotension from asphyxia can not be compensated with increased blood flow to the brain. Work in asphyxiated piglets has demonstrated that blood flow to the cerebrum was decreased and remained low during recovery with prolonged asphyxia of 60 min.[49] Poor cerebral blood flow during resuscitation occurs despite the normalization of PO_2, PCO_2, and recovery of BP. This finding has been found in human term neonates after asphyxia.[50]

Cerebral blood flow may be independent of blood pressure and may rely more to cardiac output for adequate tissue function and oxygenation.[51] Near infrared spectrophotometry studies of preterm neonates have shown no correlation between low mean blood pressure and low cerebral blood flow [52,53] Using the same technology, Tsuji *et al* correlated blood pressure to cerebral blood flow autoregulation and intraventricular hemorrhage.[47] These studies did not include term infants after asphyxia; thus, the interplay between asphyxia, blood pressure and cerebral blood flow is of yet undetermined in term asphyxiated neonates.

Metabolism, Energy and Reactive Oxygen Species

Normally, the brain's energy is produced when oxidative phosphorylation produces ATP. Hypoxia- ischemia causes a lack of oxygen, the final electron acceptor of the electron transport chain. Prolonged hypoxia can cause a reduction of blood flow below a critical ischemic threshold, leading to a further decrease in oxygen and glucose delivery.[54] Brain cells are forced to abandon oxidative phosphorylation and use anaerobic

metabolism while attempting to increase cerebral perfusion to meet their energy demands. Phosphocreatine reserves are depleted to maintain an ATP supply. When all energy reserves are exhausted, brain cells become injured or die.[55] During hypoxia, the ATP depletion in the cerebral system renders ATP dependent reuptake pumps inactive. This decreases the movement of the excitatory neurotransmitter glutamate out of the synapse into the glial cells. N-methyl-D aspartate type glutamate receptors are overstimulated and their channels are opened, and the neurons are flooded with an influx of calcium and water. Calcium overload leads to activation of proteases, lipases, and endonucleases that promote cell damage.

To restore normal cerebral function and blood flow, hypoxia must be remedied with resuscitation, reoxygenation and reperfusion. Reperfusion has been associated with an initial increase in cerebral cyclic adenosine monophosphate (cAMP) followed by a decrease to 50% of baseline.[49] Reperfusion upon reoxygenation also causes other adverse effects due to OFR formation causing further damage of the neural tissue and vasculature. Oxygen free radicals include superoxide anion, hydrogen peroxide, hydroxyl radicals and those generated by prostaglandin metabolism or xanthine, as well as nitric oxide produced by nitric oxide synthase. Free radicals can damage the cell membrane, disrupt energy production by attacking the mitochondria, and induce apoptosis.[54] The immature white matter of neonates, including oligodendrocytes progenitor cells and pre oligodendrocytes, are more vulnerable to hypoxia, ischemia, and reperfusion due to their sensitivity to OFR damage.

Diagnostic imaging has shown that term infants who undergo a short but fatal hypoxic event have relative sparing of the brain cortex matter with injury to the deep grey

matter particularly the hippocampus, lateral geniculate nuclei, putamen, ventolateral thalami, basal ganglia and dorsal mesencephalon. These grey matter structures are known to contain high concentrations of glutamate and N-methyl-D aspartate receptors.[56] A more peripheral pattern of cerebral injury is found when there is a prolonged, but less profound, hypoxic event. This pattern involves the cortex and white matter in the watershed area where blood flow is sparse. Data from animal studies have led clinicians to surmise that less severe but prolonged asphyxia may be associated with more significant brain injury and morbidity than a brief but severe insult. This must be correlated with the clinical course of the neonate. In cases where the neonate exhibits only a few aspects of the American College of Obstetricians and Gynecologists' asphyxia criteria, particularly a low Apgar and umbilical cord pH, the insult was most likely brief and intact survival is more likely.[57]

Knowledge of the theories behind cerebral cell death and hypoperfusion may help us alleviate some of the mortality and long term disabilities associated with asphyxia, such as cerebral palsy and developmental delay.

HYPOXIA- REOXYGENATION AND THE GASTROINTESTINAL SYSTEM

Impaired Perfusion

Many events leading to hypoxia have a common thread of impaired perfusion. A decrease in blood flow to organs may result in many of the short and long term morbidities found post asphyxia in the neonatal intensive care unit. The neonatal gut normally receives approximately 20% of the cardiac output.

The intestinal vascular resistance across the neonatal intestine is usually quite low. This low resistance state allows a high rate of blood flow through the intestine. Nitric oxide is thought to mediate this low resistance with a lesser vasoconstrictive balance provided by the endothelial production of the endothelin-1. Due to this low resistance state, it is difficult for the newborn intestine to respond appropriately to systemic alteration such as hypotension or hypoxemia.[58] Ischemia-reperfusion can damage the endothelium and generate OFR that alter the balance between nitric oxide and endothelin-1 to favor vasoconstriction, leading to further intestinal ischemia.[59]

Gut mucosal or transluminal necrosis can be an associated finding in neonates with asphyxia, shows histopathological changes of mucosal edema and necrosis. These changes can occur in watershed areas between the superior and inferior mesenteric arteries. As watershed areas are often damaged early during periods of low flow, watershed area necrosis gives rise to the suspicion that some cases of gut damage may be due to perinatal ischemic and hypoxic events.[60]

Mace *et al* studied the effects of 30 min of hypoxia (FiO_2=0.12) followed by 30 min of reperfusion on the mesenteric system of 2 week old piglets.[60] Using flow probes and videomicroscopy, mean BP, cardiac output and cranial mesenteric (equivalent to

superior mesenteric) artery blood flow were measured. During the short duration of hypoxia, there was no change in pH or PCO_2. Cardiac output dropped significantly with hypoxia without completely returning to baseline upon reoxygenation. Despite no significant changes in mean arterial pressure, mesenteric blood flow declined upon hypoxia but was restored with reoxygenation. Videomicroscopy confirmed that decreased mesenteric flow during hypoxia coincided with a significant vasoconstrictive response with a return to normal vessel diameter with reoxygenation. These newer experimental techniques confirmed the work by Touloukin, Alward and Karna who had previously demonstrated a decrease in blood flow to the gut in animal models of hypoxic challenge.[61-63]

In a study using a longer duration of hypoxia, (2h, FiO_2=0.07-0.15) significant shock with metabolic acidosis, systemic hypotension and decreased cardiac output was found.[64] The superior mesenteric artery (SMA) blood flow markedly decreased with no appreciable change in SMA vascular resistance. Upon reoxygenation, SMA flow increased above baseline for 5-15 min with a decrease of SMA vascular resistance. With hypoxia, mesenteric oxygen delivery was severely decreased but recovered to normoxic baseline after 4h of reoxygenation.

In the clinical realm, a study by Kemply using serial Doppler monitoring of human neonates exposed to hypoxia, demonstrated that gut perfusion was decreased or non responsive to feeds in days preceding the onset on necrotizing enterocolitis.[65] This finding may implicate decreased blood flow as a contributor to intestinal pathology.

Oxygen Free Radicals in the Intestine

Haase *et al's* study of hypoxia-reoxygenation of neonatal piglets using varying concentrations of oxygen for resuscitation demonstrated that higher concentration of oxygen may lead to increases OFR injury in the intestine.[64] Piglets that were exposed to 100% oxygen were more likely to demonstrate gross necrosis and pneumotosis intestinalis on intestinal pathology than those pigs resuscitated with 21% oxygen. The higher mesenteric oxygen delivery in the 100% oxygen group correlated with higher intestinal oxidative stress as measured by the glutathione redox ratio. A high glutathione ratio has been shown to correlate with lipid peroxidation and mitochondrial DNA damage.[64]

BLOOD PRESSURE AND BLOOD FLOW

The normal physiologic BP, as defined by normal organ blood flow, is variable in neonates. Many clinicians define "normal" BP as the range of blood pressures between the 5% and 95% for postnatal and gestational age. For the term neonate, a mean BP of 43-45 mmHg would be considered "normal" on the first day of life, increasing to 50 mmHg by 3 days of age.[24,66]

Many clinicians assume that BP and systemic blood flow are proportional. However, as BP can be low due to low resistance, low flow, or both, it can become difficult to ascertain an etiology for a neonate's hypotension or shock.

Shock is a state of dysfunctional cellular energy caused by inadequate tissue and organ perfusion resulting in decreased delivery of oxygen and metabolites as well as reduced removal of waste products. In the initial compensated stages of shock, the body alters regional blood flow to maintain vital organ perfusion at the expense of non-vital organs. If shock continues, the body is no longer able to compensate resulting in hypotension, tissue ischemia, and lactic acidosis. If treatment is delayed in this stage, shock can become irreversible with permanent organ failure and death.[24] Shock still remains a significant cause of neonatal morbidity and mortality.[67]

Ventricular output can be used to measure system blood flow but shunts through the patent ductus arteriosus or foramen ovale can complicate or alter this measure. The presence of a patent ductus arteriosus can lead to overestimation of systolic blood flow by up to 100%.[68]

Doppler measures of superior vena cava (SVC) flow have also been used to study systemic blood flow to the upper body and brain as it is not affected by ductus arteriosus

flow.[36] However, SVC flow only measures upper body flow and doesn't estimate lower body flow including flow to kidneys or intestine. As hypoxia alters the redistribution of systemic blood flow preferentially to the upper body, SVC flow may overestimate systemic blood flow in hypoxia neonates. Sick neonates often have impaired autoregulation or a redistribution of organ blood flow that alters the relationship between blood pressure, cardiac output, and organ perfusion.[69] Animal models and preliminary work of SVC flow in neonates indicate that peripheral vascular resistance, not BP, is indicative of regional blood flow post asphyxia.[34] Oxygen delivery to the tissue is influenced by cardiac output and blood flow more than BP.[67]

Another alternative to ventricular output is to measure organ blood flow specifically and directly.[68] In the case of cerebral blood flow, near-infrared spectroscopy, pulsatility index and flow velocities with Doppler ultrasound have been used. When middle cerebral artery flow measured by Doppler ultrasound techniques were compared with SVC flow and blood pressure, only SVC flow correlated to subsequent periventricular and intraventricular hemorrhages in preterm infants.[36] Whether SVC or ventricular outputs are used, only a weak relationship with BP and intraventricular hemorrhage has been found.[37,70]

Abnormal values of BP are not always associated with pathology. Clinically, neonates with hypotension can manifest changes in heart rate, decreased perfusion, decreased urine output, altered neurological status and respiratory distress. A clinical decision to treat hypotension should include evaluating the neonate's clinical response to an abnormal BP value as opposed treating just a number. A multicenter trial by Al-Alweel *et al* found a three fold variation in the occurrence of hypotension between

centres.[71] Two of the six sites had a 5-30 fold increase in use of vasopressors after adjusting for the occurrence of hypotension. A recent cohort study demonstrated that treatment with vasopressors was associated with severe periventricular hemorrhage; however, infants requiring pressor use are often very ill and have other risk factors predisposing them to bleeding in the brain.[72]

Despite many attempts to demonstrate that treating hypotension, as opposed to low blood flow or decreased cardiac output, ameliorates mortality and morbidity, no convincing literature exists to support this practice.[67]

CONCLUSION

Asphyxia can cause multiorgan dysfunction. Neonates are especially susceptible as they have little energy reserve, immature organ systems and underdeveloped free radical scavenging capabilities. As the number of organ systems damaged by asphyxia increases, so to do the rates of adverse outcomes. Many asphyxiated infants require intensive care and can be left with long term morbidity secondary to the organ dysfunction caused by perinatal asphyxia.

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CHAPTER 3

Current Medical Treatment of Neonatal Asphyxia

ASPHYXIA AND DRUGS

Neonatal asphyxia often leads to a low cardiac output state and left ventricular myocardial dysfunction which may put sick neonates at risk for decreased blood and oxygen flow to vital organs including the brain, leading to significant morbidity and mortality.[1]

This hypoxia induced cardiac dysfunction includes reduced myocardial contractility and passive dilation often in conjunction cardiac stunning with systemic hypotension and pulmonary hypertension (PHT). [1-3] As the left ventricle is dependent on blood flow returning from the pulmonary system, increased pulmonary vascular resistance with a closing ductus arteriosus can lead to a decreased systemic blood flow.[4] Some asphyxiated neonates could have low systemic blood flow without severe hypotension due to poor myocardial contractility.[5]

Stunned reperfused myocardium is sensitive to inotropic stimulation which may help with myofibril contractility to overcome some of the mechanisms of myocardial stunning.[6] Some L-type calcium channel subunits may be able to modify channel activity with β-adrenergic receptor activation and cyclic adenosine monophosphate (cAMP) dependent kinases. This allows inotropes with β-adrenergic or cAMP modification activity to increase intracellular calcium availability.[7]

The treatments for hypotension and cardiac dysfunction such as the catecholamines dopamine, dobutamine and epinephrine, may increase heart rate, cardiac function and peripheral vascular resistance at the expense of increased oxygen consumption, fluctuating blood pressures and aggravating PHT.[8] Milrinone is a specific phosphodiesterase III inhibitor that increases myocardial contractility by elevating the

intracellular cAMP and calcium.[9] It can increase cardiac stroke volume thus cardiac output and improve diastolic dysfunction.[10] Increases in cAMP in smooth muscle also cause vasodilation that can alleviate PHT.[11,12] Compared with currently used inotropes, phosphodiesterase III inhibitors are thought to increase the cardiac output with less oxygen consumption and a lower risk of arrhythmias.[13]

While there is sparse literature on the neonatal use of milrinone in congenital heart patients after cardiopulmonary bypass and for PHT, there are no studies on the use of milrinone in a neonatal model of asphyxia examining the consequent hemodynamic effects, including regional blood flow.

Current therapeutic regimens for management of the neonate after asphyxia consist of catecholamines and vasopressors.

CURRENT INOTROPES AND VASOPRESSORS USED IN THE NEONATAL INTENSIVE CARE UNIT TO SUPPORT ASPHYXIATED NEONATES

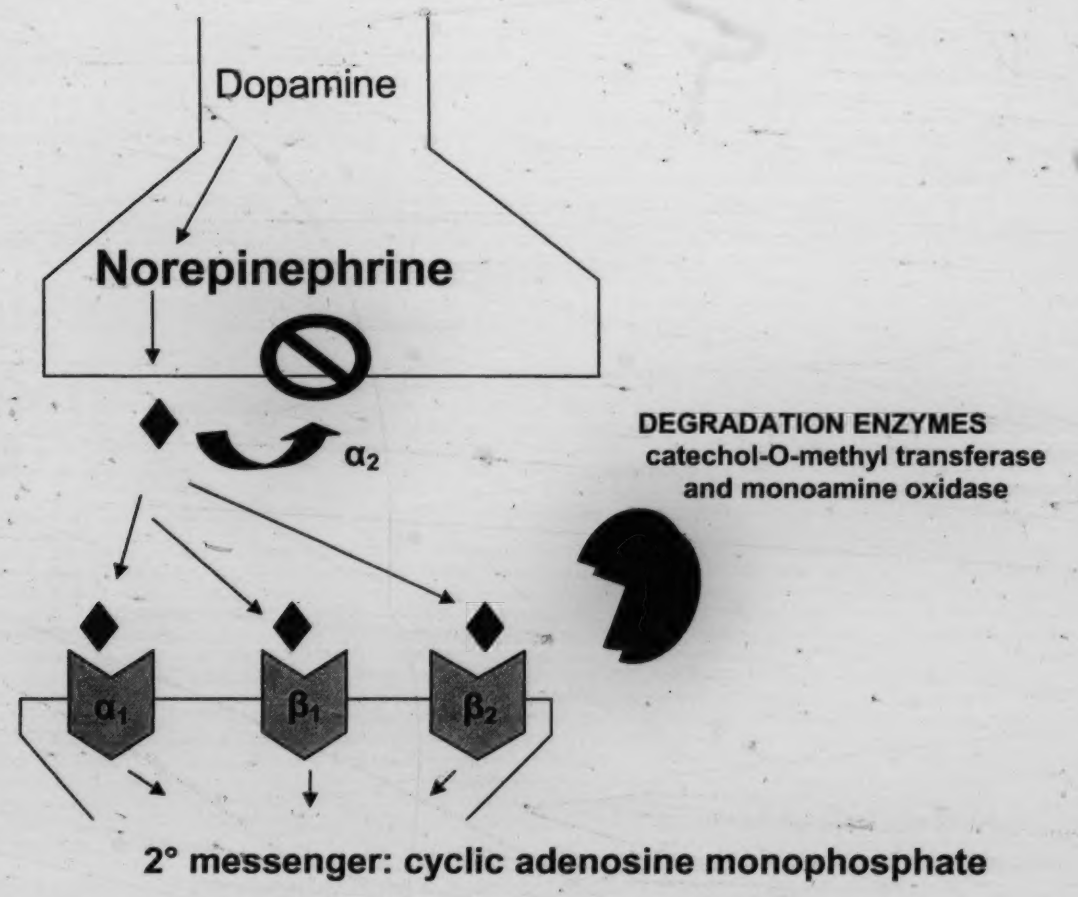
Introduction to Inotropes and Vasopressors

Dopamine, dobutamine and epinephrine are medications commonly used for cardiovascular support of the sick neonate in the intensive care unit. While they share similar mechanisms of action, they have varying effects in different organ systems. These medications can be classified by their predominant mode of action upon specific receptors within the body which can often be altered by dose.

An inotrope is a medication that increases the cardiac contractility of the heart whereas a chronotrope will increase the heart rate. A vasopressor is a medication that produces vasoconstriction contradictory to a vasodilator. A lusitrope will improve diastolic function of the heart. Many of these medications work within the autonomic system to alter sympathetic nervous system synapse mechanisms within the cardiac or smooth muscle of vessels. Dopamine, dobutamine and epinephrine are categorized as catecholamines as their chemical structures contain a catechol and amine group. They are sympathomimetics as they directly stimulate the sympathetic nervous system by binding receptors at the synapse and activating second messenger systems.

In the sympathetic nervous system synapse, naturally occurring dopamine is converted to norepinephrine and released from the presynaptic neuron into the synapse to stimulate post synaptic adrenergic receptors. Adrenergic receptors are responsible for initiating the cascade of reactions responsible for the actions of these medications throughout the body. Catecholamines are degraded quickly within the synapse by the body enzymes, catechol-O-methyl transferase and monoamine oxidase (Figure 3-1).

FIGURE 3-1 : SYMPATHETIC NERVOUS SYSTEM SYNAPSE



α_1, α_2, β_1, and β_2 are different subtypes of adrenergic receptors

Sympathomimetics can act directly, indirectly or through a mixed pathway. Direct agents, such as dobutamine, epinephrine and dopamine (to some extent), mimic elements of norepinephrine as agonists at adrenergic receptors without interaction at the presynaptic neuron. Indirect sympathomimetics, including amphetamines and dopamine at inotropic doses, enhance the actions of endogenous norepinephrine and do not bind adrenergic receptors themselves.

Alpha-1 adrenergic receptors are found predominantly in arterioles and veins and cause vasoconstriction. Alpha-2 adrenergic receptors can inhibit norepinephrine release from the presynaptic neuron and decrease sympathetic outflow. Beta-1 adrenergic receptors increase heart rate and contractility. Beta- adrenergic receptor agonists bind to Gs-coupled β- adrenergic receptors and activate adenylyl cyclase to produce cAMP. Cyclic AMP can in turn activate protein kinase A to phosphorylate the troponin complex, phospholamban, and L-type channels. L-type channels increase calcium influx and increase troponin complex responsiveness and contractility. Elevated cAMP levels also increase the rate at which diastole can occur, allowing increased sequestration of calcium into the sarcoplasmic reticulum via phospholamban and protein kinase A. However, the β-adrenergic receptor must be functional and not downregulated for these actions to occur. Beta-2 adrenergic receptors cause vasodilation (except in the brain and skin) as well as relaxation of lung bronchioles. The dopamine-1 receptor is postjunctional and causes vasodilation in vascular beds such as the adult renal circulation, mesentery, brain and coronaries. Dopamine-2 receptors are both pre and post junctional and can inhibit presynaptic norepinephrine release and inhibit adenylyl cyclase.

Changes in cardiovascular adrenergic receptors expression can be altered by critical illness, extended catecholamine use, or relative adrenal insufficiency as well as gestational age and maturity. [14] In the failing myocardium, there is a downregulation of β-1 adrenergic receptors. Uncoupling of β-2 adrenergic receptors from adenylyl cyclase, increased activity of inhibitory guanine nucleotide binding protein, and decreased adenylyl cyclase and cAMP production have also been described in a failing heart.[8]

Glucocorticoids have been found to regulate expression of adrenergic receptors. Mineralocorticoids (found in hydrocortisone) and pharmacologic doses of glucocorticoids can regulate the second messenger system downstream of the adrenergic receptor by increasing intracellular calcium to increase myocardial contractility.[15] It is hypothesized that in the critically ill infant, an immature and stressed hypothalamic- pituitary adrenal axis is not able to increase endogenous steroids at a great enough rate to prevent adrenergic receptor downregulation. Decreased catecholamine efficacy or resistant hypotension can result.[16]

Although literature exists for the treatment of hypotension in premature neonates, there is almost no evidence as to what drug or dose to use in the term asphyxiated infant suffering from hypotension or cardiac dysfunction.[17] Dopamine, dobutamine, and epinephrine are the current medications commonly used to treat cardiovascular instability in neonates after hypoxia-reoxygenation.

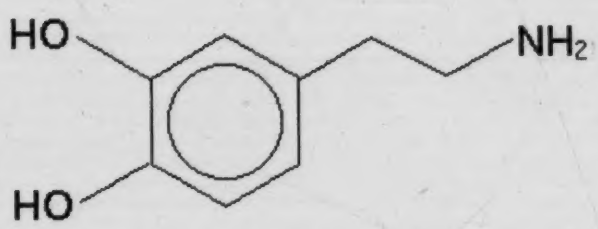
Dopamine (Figure 3-2A)

Dopamine is one of the most common medications used in the neonatal intensive care unit to for cardiovascular support of sick neonate. It is an endogenous catecholamine precursor of norepinephrine with sympathetic properties. It can act directly through the stimulation of dopaminergic and adrenergic receptors. Dopamine can also act indirectly by stimulating adrenergic receptors through conversion to norepinephrine in the sympathetic nerve endings.[18] The half life of dopamine is 2 min in healthy adult and pediatric patients. The effects of dopamine are usually lost within 20 min of discontinuing an infusion. In a study of 11 critically ill neonates receiving 5-20 μg/kg/min of dopamine, the elimination half life was found to be 6.9 min with significant variation between sick infants.[19] Dopamine is metabolized in the plasma, the kidneys and the liver.[20] Clearance varies with gestational age and can be as long as 60 min in critically ill neonates.[21]

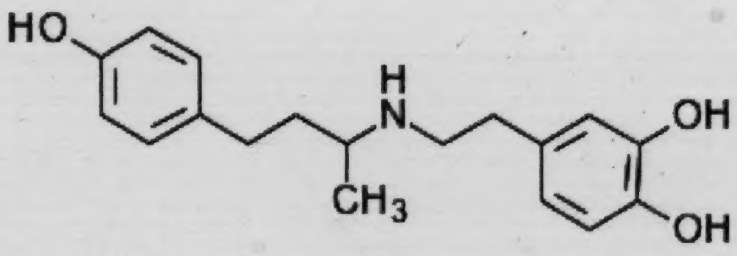
The effects of dopamine at different doses, different gestations, and ages are currently debated. Conventional teaching suggests that at a dose of 1-5 μg/kg/min dopaminergic receptors on the kidneys are activated to increase renal blood flow and increase urine output. At a moderate doses of 5-10 μg/kg/min, dopamine becomes inotropic with a β-1 effect predominating with 50% of myocardial contractility relying on norepinephrine stores to interact with the β-1 adrenergic receptor.[20] At dose of 10-20 μg/kg/min, dopamine delivers more of an α-1 effect with vasoconstriction. This teaching has been derived from healthy adult studies and pharmacokinetics.[22] The variable effects at different doses remains controversial.

FIGURE 3-2: STRUCTURES OF DOPAMINE, DOBUTAMINE AND EPINEPHRINE

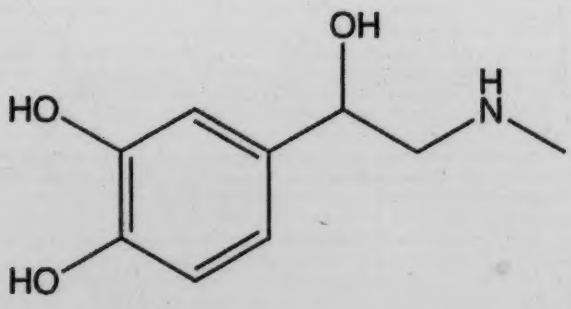
A) Dopamine



B) Dobutamine



C) Epinephrine



Interpatient variability, found in neonates with differing underlying pathophysiology can result in varied responses to the same dose in different patients.[13]

Neonatal research is conflicting as to whether the activity of the dopamine-1 receptor is even mature enough to respond appropriately despite its presence in the newborn vasculature. In a study of chronically instrumented healthy unanaesthetized piglets, fenoldopam, a selective dopamine -1 receptor agonist, produced vasodilation and increased superior mesenteric blood flow.[23] This result indicated that dopamine-1 receptors may be present and functional in the mesentery of neonatal piglets. However, no change in mesenteric vasodilation was found with dopamine (dose range 2-32 μg/kg/min).[23] Mesentery flow increased with 32 μg/kg/min which coincided with an increase in blood pressure. The authors of this study hypothesized the coexistence of both dopamine-1 and α-adrenergic activity in the neonatal mesenteric vasculature with the balance negating dopamine's effect. This hypothesis is supported by the work of Ferrara *et al* who found that, depending on the maturity of the gut, different mesenteric adrenergic receptors are present.[24] Ferrera *et al* demonstrated that in 1 day old term piglets α_{1A} and α_{2B} adrenergic receptors predominate whereas α_{1B} and α_{2B} adrenergic receptors are present in older piglets.

Despite variations in animal model, anesthesia, length of hypoxia and subsequent stabilization, modes of blood flow measurement and dopamine dosing, animal studies have demonstrated that only high doses of dopamine increase cardiac output during normoxia, but not during hypoxia.[25-28] Lower doses of dopamine (2-10 μg/kg/min) did not alter MAP, PAP, or cardiac output.[29] High-dose dopamine increases the MAP and PAP but negatively impacts on the MAP/PAP ratio leading to concerns of right to left

shunting and aggravating tissue hypoxia.[25] Higher doses of dopamine can decrease mesenteric vascular resistance, especially during hypoxia which may decrease mesenteric α- adrenergic receptors or increase dopaminergic receptors.[28,30,31] Animal studies have demonstrated little effect on the renal vasculature at any dose of dopamine.[23] Interestingly, dopamine doses have been found to consistently elevate MAP in human neonates at doses of ≥10 μg/kg/min. Piglets may metabolize dopamine differently and have different expressions of dopaminergic or adrenergic receptors, thus explaining why 32 μg/kg/min is needed to increase piglet MAP compared to10 μg/kg/min in human neonates. Some piglet studies use healthy animals, without cardiovascular compromise which may also alter the dose at which effects of dopamine are seen. While important, these results could not be generalized to the population of sick neonates in whom dopamine is often administered.

In the clinical realm, dopamine is one the most commonly studied cardiovascular drugs. Dopamine has been reported to increase urine output and renal blood flow in the neonate.[32,33] As neonates have a normal physiologic increase in urine output and blood pressure in the first 48 h, support for dopamine renal effects can not be validated as these studies did not include a control group for comparison and made measurement within the first 48 h. Furthermore, the increase in glomerular filtration rate and urine output with low-dose dopamine (1-2.5 μg/kg/min) was not confirmed by Cuevas *et al.*[34] A study in preterm infants demonstrated that dopamine doses that increase blood pressure were also associated with increased vascular resistance and decreased cardiac output.[35]

A Cochrane Database systemic review of dopamine use in term babies with suspected perinatal asphyxia found only one randomized controlled study that compared

dopamine (2.5 μg/kg/min) to placebo in this population.[36] No significant differences were found between the two groups with respect to mortality or morbidity despite increases in MAP.[37]

Despite widespread use of dopamine in the neonatal intensive care unit, there is still a lack of convincing evidence that its use yields any difference in mortality or long term morbidity. Occasionally, hypotension and poor blood flow do not respond to dopamine or the underlying cardiovascular pathophysiology is deemed incompatible with dopamine use. In these cases, alternative inotropes or vasopressors are used.

Dobutamine (Figure 3-2B)

Dobutamine is a synthetic catecholamine that acts directly on the β-adrenergic receptor. Dobutamine, as an inotrope, increases cardiac contractility and also has chronotropic effects that may increase heart rate at higher doses. There is a small β-2 adrenergic effect that can cause weak vasodilation as well as a mild α-1 effect which hypothetically can increase MAP at high doses. There is no dopaminergic stimulation associated with dobutamine and it does not rely on the release of endogenous norepinephrine supplies. It has a half life of 2 min and loses hemodynamic effects 10 min after infusion discontinuation. Plasma clearance does not depend on gestational age. It is metabolized in tissues and the liver. Doses in the neonatology range between 2.5-20 μg/kg/min. Compared to epinephrine, it provides inotropic effects with less myocardial oxygen consumption and a decreased rate of arrhythmias.[38] Chronic stimulation of the adrenergic receptors eventually leads to downregulation and desensitization.[38]

In microsphere studies of term normoxic anesthetized piglets, dobutamine increased cardiac index, MAP, and heart rate at a dose of 15 μg/kg/min.[39] Dobutamine dose-dependently caused a mild increase in cerebral and cardiac blood flow with little effects on renal blood flow. High-dose dobutamine produced a mild reduction in intestinal blood flow. To negate the effects of anesthesia and acute instrumentation, Cheung *et al* administered 15 min of randomly assigned doses of dobutamine (5-50 μg/kg/min) to unanaesthetized chronically instrumented piglets.[39] This protocol also included a 2h infusion of 10 μg/kg/min of dobutamine. Short term dobutamine increased cardiac output via chronotropy but did not increase stroke volume. There was an increase in pulmonary arterial pressure and renal vasoconstriction at the highest dose. There was no increase in renal blood flow. A 2h infusion of dobutamine at 10 μg/kg/min resulted in an increase in cardiac index and stroke volume with a decrease in peripheral vascular resistance. Tachycardia was transient and returned to baseline after 60 min of infusion. It is hypothesized that the normalization of heart rate may be due to desensitization of the β-adrenergic chronotropic receptors or vagal stimulation secondary to the continued increase in cardiac output.[40] Stroke volume may increase with prolonged infusion as a response to a decrease in peripheral vascular resistance. These studies used healthy piglets that had not undergone asphyxia or reoxygenation and had not manifested any cardiovascular instability.

To mimic the clinical scenario of neonatal asphyxia and resuscitation, Al-Salam *et al* administered dobutamine (5, 10 or 20 μg/kg/min) to anesthetized acutely instrumented piglets who had undergone 2h of hypoxia (FiO_2=0.1-0.15) and 2h of reoxygenation prior to receiving dobutamine.[41] Compared to controls, dobutamine at 20 μg/kg/min improved

cardiac index, stroke volume and systemic oxygenation without increasing oxygen consumption. All doses decreased pulmonary vascular resistance but did not affect systemic vascular resistance, MAP or PAP. No significant increases in regional perfusion were found.

In a small study of 6 asphyxiated term neonates, noninvasive monitoring demonstrated that dobutamine (10 μg /kg/min) increased cardiac output and heart rate.[42] Lower dose dobutamine (2.5-7.5 μg/kg/min) also increased cardiac output without significant changes in MAP in a study of 13 sick neonates.[43] The threshold plasma concentration of dobutamine resulting in increased cardiac output, was 39±8 ng/mL and the mean plasma clearance rate was 90±38 mL/min/kg.[43] This study calculated the plasma threshold levels needed to change the "cardiovascular response" for each patient but did not present their definition of "increased cardiac output."

There is a small body of data to suggest that in addition to improving cardiac output and increasing heart rate at higher doses, the α-adrenergic effect of dobutamine may be exaggerated in human neonates leading to an increase in blood pressure.[44,45]

Epinephrine (Figure 3-2C)

Epinephrine is an endogenous catecholamine that acts directly on the adrenergic receptors. It is physiologically released during times of stress from the adrenal gland. It has effects on α_1, α_2, β_1 and β_2 adrenergic receptors. The half life of epinephrine is 2 min. As epinephrine acts directly on the adrenergic receptors, it is not dependent on an endogenous supply of catecholamines as dopamine is. Epinephrine is believed to have different mechanisms of cardiovascular support depending on the dose. In neonates, at

low doses (0.05- 0.2 μg/kg/min) epinephrine acts predominantly on the β_1 and β_2 adrenergic receptors and has an inotropic action to increase cardiac contractility. The range of moderate dose epinephrine in a neonate is not well defined but is generally regarded as 0.2 -0.5 or up to 1.0 μg/kg/min. At this dose the vasoconstrictive effect of α_1 adrenergic receptor stimulation becomes more apparent and starts to counter the β_1 inotropic effect. However, the resultant blood flow to the brain and heart can be increased. At the higher end of this range, vasoconstriction can become intense, tachycardia occurs, blood flow to the gut and kidneys decrease and increased oxygen consumption occurs. This may put additional stress on an already compromised neonatal heart and peripheral tissues. High- dose epinephrine (> 1.0 or 2.0 μg/kg/min) is only used as a treatment of last resort and is regarded as a continuous resuscitation measure. At this dose, severe vasoconstriction occurs in a neonate with loss of significant blood flow to peripheral organs in order to preserve flow to the heart and brain. Disorganized energy utilization with decoupling of oxidative phosphorylation and excessive vasoconstriction may lead to a decreased cardiac output at epinephrine doses >2 μg/kg/min.

Studies in normoxic and hypoxic newborn animals have demonstrated that epinephrine increased cardiac index (0.2-3.2 μg/kg/min) and decreased systemic vascular resistance at lower doses.[26-28] High-dose epinephrine increased PAP but not MAP and not enough to alter the MAP/PAP ratio. There was no significant decrease in mesenteric vascular resistance.[28] With stimulation at both α and β adrenergic receptors, epinephrine would be a more appropriate agent for a hypoxic neonate if the "same effects are present in the human infant" as those seen in a piglet.[28] Interestingly, clinical experience demonstrates that asphyxiated neonates do not tolerate the extreme vasoconstriction

caused by epinephrine doses of > 1 μg/kg/min,[46] which was not observed in piglet studies.[26-28] Therapeutic doses of epinephrine differ between humans and piglets.

Hyperglycemia and increased blood lactate become evident at the upper end of moderate-dose epinephrine (0.5-1 μg/kg/min).[47] Increased plasma lactate levels may be associated with poor tissue perfusion secondary to vasoconstriction.[18] Stimulation of β_2 adrenergic receptors and/or phosphofructokinase by epinephrine causes skeletal muscle glycogenolysis and hepatic glycogenolysis and gluconeogenesis allowing plasma glucose and lactate levels to rise. With prolonged epinephrine infusions, piglet myocardium has demonstrated neonatal sarcolemmal rupture, increased cytoplasmic calcium deposits, and decreased myocardial compliance.[48] Asphyxiated rats had decreased survival and increased left ventricle ischemic contractures when given epinephrine for cardiac arrest.[49]

Because of the paucity of literature for the use of epinephrine in human neonates and potential risks of high-dose epinephrine, neonatologists tend to use epinephrine for refractory hypotension in neonates.[50] Much on the literature is in abstract form and involves preterm infants instead of asphyxiated term infants.[47,51-53] In the study by Phillipos *et al*, epinephrine and dopamine both increased heart rate and blood pressure with no significant effect on left or right ventricular outputs.[51] A 2004 Cochrane Database systematic review of epinephrine for prevention of morbidity and mortality in preterm infants with cardiovascular compromise found only one study and one abstract to fulfill the randomized controlled trial criteria.[47,51,54] This review concluded that there was insufficient data to make recommendations for the use of epinephrine in the preterm neonatal population.[54]

In 2005, Pellicer compared the effect of dopamine (2.5-10 μg/kg/min) and epinephrine (0.125-0.5 μg/kg/min) on cerebral hemodynamics using near infrared spectrophotometry in 60 premature neonates with hypotension in a randomized double blinded fashion.[47] Significant increases in blood pressure and heart rate were found with no significant difference between dopamine and epinephrine except for significant tachycardia with high-dose epinephrine. Cerebral blood flow increased significantly in the more premature infants (< 28 weeks) with epinephrine and in the more mature infants (28-32 weeks) with dopamine. No cerebral vasoconstriction was found. Epinephrine was more likely to result in hyperglycemia and increased plasma lactate.[53] The downfall of this study is the lack of cardiac output or systemic flow measures to correlate to blood pressure or cerebral blood flow; thus, we still do not know if the drugs acted as inotropes to improve cardiac contractility and increase organ blood flow or as vasopressors to change blood pressure and cause increased cerebral blood flow in the presence of impaired cerebral autoregulation.

Traditionally, inotropic agents and vasopressors have been used to support neonates post asphyxia-reoxygenation (Table 3-1). The use of catecholamines has several drawbacks, including increased myocardial oxygen consumption and afterload, tachycardia, and promoting arrhythmias. In addition, adrenergic receptors may be downregulated in critically ill neonates.

Phosphodiesterase inhibitors such as milrinone have been used in selected neonatal populations to support a low cardiac output or decrease pulmonary hypertension. There is currently no literature on the use of milrinone for low cardiac output, pulmonary hypertension and cardiovascular instability in the hypoxic-reoxygenated neonate.

TABLE 3-1: INOTROPES USED FOR NEONATAL CARDIOVASCULAR INSTABILITY

Drug	Category	Mode of action	Theoretical Cardiovascular Effects	Infusion Dose (µg/kg/min)
Dopamine	Inotrope/ Vasopressor	- β_1 agonist - Mild α_1 agonist - Dopaminergic	*Low-Dose*: *May* increase mesenteric perfusion, *may* increase urine output *Mid-Dose:* Increase myocardial contractility *High-Dose*: Vasoconstriction	*Low-Dose:* 2-5 *Mid-Dose:* 5-10 *High-Dose*: 10-20
Dobutamine	Inotrope	- β_1 agonist	Increase myocardial contractility Mild vasodilator	5-20
Epinephrine	Inotrope/ Vasopressor	- α_1, α_2, β_1 agonist - Mild β_2 agonist	*Low-Dose*: Increase myocardial contractility, increase cardiac output *Mid-Dose:* Vasoconstrictor more than inotrope *High-Dose*: Vasoconstriction	*Low-Dose:* 0.05-0.2 *Mid-Dose:* 0.2- 0.5 *High-Dose*: > 1.0
Milrinone	Inotrope Lusitrope	- Phosphodiesterase III inhibitor	Increase myocardial contractility and cardiac output Decrease pulmonary and systemic vascular resistance	*Load:* 25-75 µg/kg *Infusion:* 0.25-0.75

CONCLUSION

Despite the lack of literature and evidence, dopamine, dobutamine and epinephrine continue to be used frequently for the management of cardiovascular instability in neonates after asphyxia. Consideration must be given to the status of systemic blood flow, cardiac output, and blood pressure in order to tailor inotropes and pressors to the condition of term neonate. Other medications to support the cardiovascularly compromised asphyxiated neonate, including phosphodiesterase III inhibitors, require to be investigated.

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CHAPTER 4

Milrinone as an Alternative Medication to Support Cardiovascular Compromise After Neonatal Hypoxia-Reoxygenation

MILRINONE

Milrinone, (1,6-dihydro-2-methyl-6-oxo-[3,4'- bipyridine]-5-carbonitrile lactate) is a bipyridine derivative (Figure 4-1). It is a phosphodiesterase (PDE) type III inhibitor that disrupts the breakdown of intracellular cyclic adenosine monophosphate (cAMP) in cardiac muscle and vascular smooth muscle. The elevation in cAMP levels increases myocardial contractility by elevating the calcium concentration.[1] Milrinone can cause vasodilation in both the systemic and pulmonary vasculature.

Increased cAMP levels can activate protein kinase A to phosphorylate the troponin complex and L-type channels. L-type channels increase calcium influx, making the troponin complex more responsive with increased contractility. Milrinone increases myocardial contractility through a cyclic AMP-mediated increase in trans-sarcolemmal calcium flux, thus its role as an inotrope (Figure 4-2). Increased cAMP and protein kinase A also increases the phosphorylation of phospholamban leading to an increase in the sequestration of calcium into the sarcoplasma reticulum as well as an improvement in actin-myosin disassociation during diastole.[2] This can improve diastolic function (lusitropy). Milrinone also removes free intracellular calcium needed for vascular smooth muscle contraction, causing peripheral vasodilation.

Phosphodiesterase inhibitors may also increase calcium via a reverse activity of the sodium-calcium exchanger allowing an increased influx of extracellular calcium.[3] As the calcium release from the sarcoplasmic reticulum is immature in neonates, these sources of extracellular calcium may be important for a neonate during times of cardiac dysfunction.

FIGURE 4-1: MILRINONE STRUCTURE

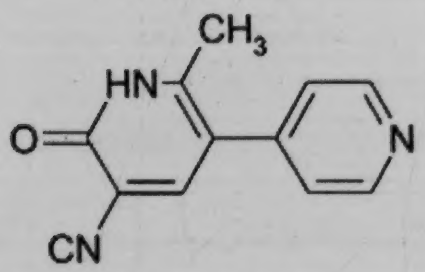
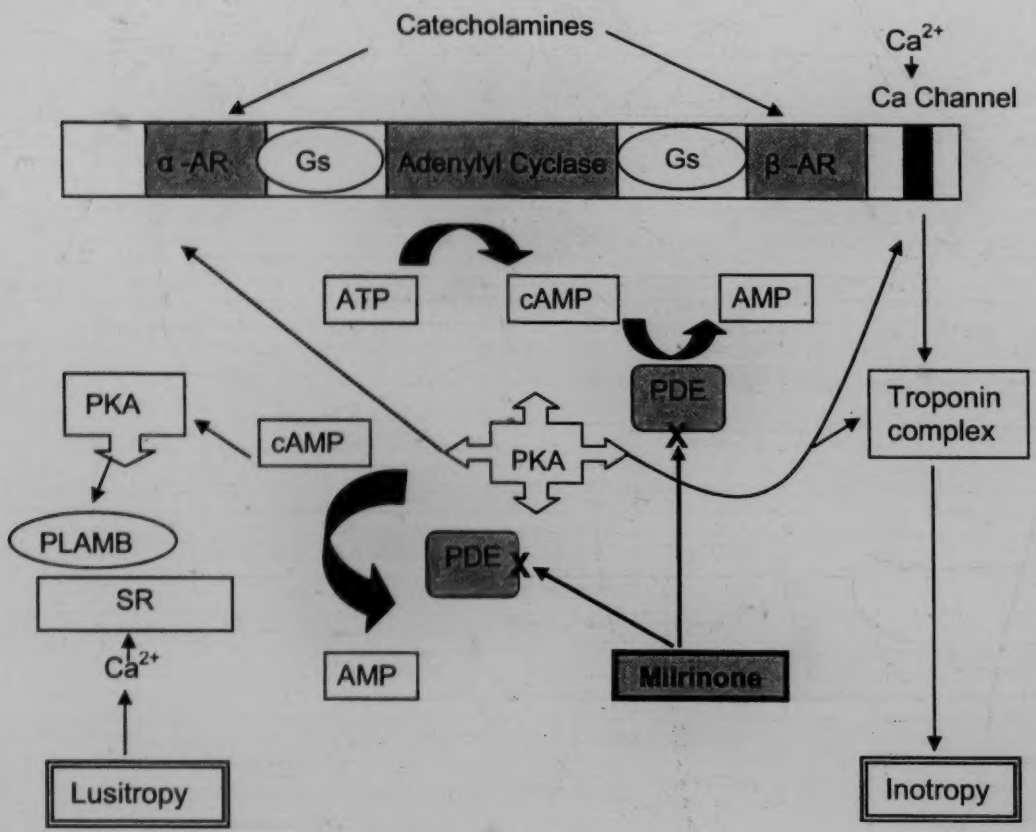


FIGURE 4-2: MECHANISM OF ACTION OF MILRINONE IN THE CARDIOMYOCYTE



α - AR:	alpha adrenergic receptor
β - AR:	beta adrenergic receptor
Gs:	G protein
Ca^{2+}:	calcium ion
ATP:	adenosine 3',5' triphosphate
cAMP:	cyclic adenosine monophosphate
AMP:	adenosine monophosphate
PDE:	phosphodiesterase inhibitor
PKA:	protein kinase A
PLAMB:	phospholamban
SR:	sarcoplasmic reticulum

Clinically, milrinone can increase cardiac stroke volume thus cardiac output and improve diastolic function.[4,5] Milrinone's ability to cause smooth muscle relaxation can alleviate pulmonary hypertension, but may also decrease systemic arterial pressure as well, the magnitude of which are debated in the literature.[6-9] Interestingly, the improvement in ventricular function is not associated with increased myocardial oxygen consumption.[7,9]

Compared with currently used inotropes, PDE III inhibitors are thought to augment cardiac output without increasing oxygen consumption or aggravating dysrhythmias.[10] Possibly due to its site of action distal to the β-adrenergic receptor, the myocardial contractile response of milrinone seems to be preserved with ongoing use, compared to the attenuation found with some other inotropes in very sick or asphyxiated infants.[8]

Milrinone has been used to improve hemodynamics in neonates and children after cardiac surgery or with septic shock.[7,11] It has been used in preterm neonates to prevent low systemic blood flow as well as for refractory pulmonary hypertension.[12] Due to the increasing interest in the use of milrinone for cardiac dysfunction, a Cochrane Systematic Review was initiated in 2004.[13]

Pharmacology of Milrinone (Table 4-1)

Based on the sparse pediatric literature, milrinone administered at a loading dose of 25-75 μg/kg followed by an infusion of 0.25 to 0.75 μg/kg/min demonstrates beneficial hemodynamic effects without adverse side effects in the pediatric population. However, neonatal dosing is extrapolated from pediatric dosing studies.[7,11,14-17] Current

pediatric drug dosing references indicate that milrinone is excreted unchanged in the urine.[18] The volume of distribution for infants is 0.9±0.4 L/kg, with a half life of 3.15±2 h and a clearance of 2.6 mL/kg/min.[19] With the exception of clearance, pharmacokinetics are derived from infants older than 1 month of age, not neonates.

Zuppa *et al* specifically looked at the pharmacokinetics of milrinone in 16 neonates.[19] These patients had undergone surgery for hypoplastic left heart syndrome. It was found that in the presence of renal impairment, clearance of milrinone was impaired at 0.4 mL/kg/h compared to 2.6 mL/kg/min at steady state. The authors recommended that during states of renal impairment, an infusion rate of 0.2 μg/kg/min should be considered. Apart fro the small sample size, the loading dose of milrinone (100-250 μg/kg) that was delivered into the cardiopulmonary bypass circuit could have greatly complicated the pharmacokinetics of milrinone.

Another pharmacokinetic study of children receiving milrinone after cardiac repair determined that clearance was a linear function of age equal to 2.42 ml/kg/min*[1+0.0396*age].[17] This study included 46 neonates and found that the clearance of milrinone in neonates was less than 25% of that in children, but still faster than adults. In neonates a constant-rate infusion will take much longer to approach steady-state levels compared to infants, with a loading dose required for the rapid achievement of a therapeutic blood concentration. However, for the same infusion rate, the steady-state concentration will be higher in neonates than older patients.[17] A study in children aged 1 month-13 years recovering from cardiac surgery demonstrated steady state plasma levels at 113±39 to 206±74 ng/mL depending on the milrinone dose (50-75

μg/kg load: 0.25-0.75 μg/kg/min infusion). The elimination half life in infants was longer than in older children (3.15±2 vs. 1.86±2 h respectively).[20]

Lyndsay *et al* performed a pharmacokinetic study of milrinone in 12 patients with septic shock between ages 6 months and 18 years. These patients were older and were all on at least 2 other inotropes (dobutamine, dopamine, epinephrine, norepinephrine). The average steady state plasma concentration was 81±39 ng/mL. The median half life was 1.47h.[11]

The mean milrinone plasma concentration to achieve a significant hemodynamic effect has been determined to be 140- 235 ng/mL leading to the conclusion that an approximate therapeutic target for milrinone would be 200 ng/mL.[7,16] Only 10 patients in these studies were neonates, with an improvement of cardiac index at 140 ng/mL.[7]

Studies of plasma concentration and therapeutic effect have not been well established and not addressed at all in the neonatal population.

TABLE 4-1: PHARMACOLOGY OF MILRINONE

Reference	Population	Milrinone Regiment	Clearance (mL/kg/min)	Terminal Elimination Half Life (hr)	Steady State Serum Level (ng/mL)	Recommend Dosing (µg/kg/min)
Zuppa *et al.*[19] *Anesth Analg ;* 2006	16 neonates post cardiac surgery for hypoplastic left heart	100 or 250 µg/kg in cardiopulmonary bypass circuit	2.6 mL/kg/min		Max level =287-662	With ↓ renal function = 0.2 to achieve 200 ng/mL level
Bailey *et al.*[17] *J Pharmacokinet Pharmacodyn ;* 2004	Subset of 46 neonates post cardiac surgery	25 µg/kg load + 0.25 µg/kg/min OR 75 µg/kg load + 0.75 µg/kg/min	2.42 mL/kg/min [1+0.0396*age]			Need to load neonates due to longer time to reach steady state
Bailey *et al.*[16] *Anesthesiology;* 1999	20 children ages 3 -22 mos No neonates	50 µg/kg load + 0.5-0.7 µg/kg/min	2.5 *weight(kg)* [1+0.058*age(mos)]		235 - achieve clinical effect	Infants- 3 µg/kg/min for 30 min + 0.5 µg/kg/min
Ramamoorthy *et al.*[20] *Anesth Analg*;1998	19 children ages 1 mos-11 years post cardiac surgery No neonates	50 µg/kg load + 0.5 µg/kg/min OR 75 µg/kg load + 0.75 µg/kg/min	Infants < 1yr 3.8 mL/kg/min	3.15	Low = 113 High = 206	
Lyndsay *et al.*[11] *J Pediatr*; 1998	12 patients ages 3 mos-18 years with septic shock No neonates	50 µg/kg load + 0.5 µg/kg/min	11± 9.6 mL/kg/min	2.88	81	

Side Effects

Milrinone can produce a slight shortening of the atrioventricular node conduction time that can cause an increased ventricular response rate and may predispose patients to arrhythmias.[21] Thrombocytopenia has been described with amrinone and, to a lesser extent, milrinone. No increase in the incidence of clinically significant tachycardia or dysrhythmias has been reported in pediatric milrinone studies. There has been no reports of significant thrombocytopenia except in Ramamoorthy's study of children after cardiopulmonary bypass (58% with milrinone vs. 25% without milrinone).[20] The results of Ramamoorthy's research have not been duplicated in other pediatric studies. Other milrinone side effects described in adult studies include hypokalemia, abnormal liver enzymes, tremor and, rarely bronchospasm.[22]

Pediatric and Neonatal Studies of Milrinone

Most studies of milrinone in the infant population involve treating low cardiac output syndrome in patients after congenital cardiac surgery. Low cardiac output syndrome is a fall in cardiac output that occurs about 6-18 h after cardiac surgery occurring in approximately 25% of pediatric patients after cardiac surgery.[14] This patient population can also have increased systemic and pulmonary vascular resistance.[23] Postoperative management concerns for pediatric cardiac patients are similar to those for asphyxiated term neonates and include improving cardiac contractility, decreasing diastolic dysfunction, reducing afterload and pulmonary vascular resistance.

In 1995, Chang *et al* described the hemodynamics of 10 neonates with post operative low cardiac output syndrome treated with milrinone.[7] Low cardiac output

syndrome was defined as a cardiac output of less than or equal to 3.0 L/min/m^2, despite adequate filling pressures (defined as a left atrial pressure > 8 mm Hg). The neonates were loaded with 50 μg/kg IV milrinone over 15 min followed by 30 min of 0.5 μg/kg/min IV milrinone within 6 h of returning from surgery. Patients also received a concomitant dopamine infusion at 3-7.5 μg/kg/min and received 5 to 10 mL/kg of 5% albumin to maintain an adequate preload. Although all patients experienced an increase in heart rate, especially during milrinone loading, no patient increased their heart rate greater than 15% above baseline. One patient had some premature atrial beats during milrinone load, but no other dysrhythmias were found. Tachycardia has been observed with high-dose milrinone in adult studies but less than that observed with dobutamine.[24] Avoiding tachycardia is important to the postoperative cardiac patient as it has been shown to be correlated with postoperative ischemia after cardiac surgery.[25]

In Chang *et al's* study, right and left atrial pressures decreased with milrinone infusion.[7] Mean arterial pressure (MAP) decreased with the loading dose (from 66±12 to 57±10 mm Hg, $p<0.01$) but was maintained during the infusion stage (59±12 mm Hg), although still lower compared with baseline ($p<0.05$). Two patients had a decrease in MAP > 20% during the infusion stage but did not require further medical intervention. During the treatment of shock or the use of inotropes, adequate preload should be ensured. There is a correlation between low preload and hypotension during administration of PDE inhibitors.[26] Pulmonary arterial pressure (PAP) decreased slightly with the greatest effect in patients with a PAP > 25 mmHg. The cardiac output increased > 20% in all but one patient. There was a decrease below baseline in pulmonary and systemic vascular resistance with an increase in both right and left stroke work index.

Chang *et al* used rate pressure index (heart rate x systolic blood pressure) as an indirect measurement of myocardial oxygen consumption and found that it was not increased. The decrease in systemic vascular resistance may reduce systolic wall stress leading to less myocardial oxygen consumption.

This study had a very small study size that may have limited its ability to detect side effects. Thrombocytopenia was not commented on. Each neonate served as their own control but there were no other children with low cardiac output on conventional inotropes to serve as controls. Many neonates are treated with milrinone for days. However, Chang *et al's* study monitored short term hemodynamic effects within hours of milrinone administration.

The Prophylactic Intravenous Use of Milrinone After Cardiac Operation in Pediatrics (PRIMACORP) study by Hoffman *et al*, evaluated the safety and efficacy of the prophylactic use of milrinone in pediatric patients aged 2 days to 7years (median 3 months) at high risk for development of low cardiac output syndrome after undergoing cardiac surgery. [14] This study was a multicenter, randomized, double-blind, placebo-controlled, parallel treatment study with three treatment groups consisting of: (1) low-dose milrinone (25 μg/kg IV bolus over 60 min followed by a 0.25μg/kg/min IV infusion for 35h), (2) high-dose milrinone (75 μg/kg bolus and then 0.75μg/kg/min IV infusion for 35h), or (3) placebo. High-dose milrinone significantly reduced the risk the development of low cardiac output syndrome compared with placebo, with a relative risk reduction of 55% ($p<0.05$) in 238 treated patients. Mean arterial blood pressure decreased by 9% in the milrinone group at the end of the bolus but was not significantly different from placebo after 12h of infusion. The heart rate was 10 beats/min higher in milrinone groups.

The high-dose milrinone group significantly decreased left atrial pressure. There was no significant difference in thrombocytopenia (high = 2.6%, low = 8.8%, placebo = 7.4%) between groups with an incidence significantly lower than that reported in Ramamoorthy's study (58%).[20] The PRIMACORP study concluded that the prophylactic use of high-dose milrinone reduced the risk of low cardiac output syndrome after pediatric congenital heart surgery without evidence of adverse side effects such as hypotension, thrombocytopenia, and dysrhythmias. This study did not measure cardiac output and milrinone was used prophylactically instead of as a treatment for low cardiac output syndrome.

Duggal *et al* assessed the direct myocardial effects of milrinone in infants with low cardiac output syndrome post cardiac surgery.[27] This was a heterogeneous population of 15 patients between 1 week and 16 months of age who received milrinone (0.3-0.6 μg/kg/min- no bolus) for 18-24h for a low cardiac output state. Cardiac function was assessed by Doppler derived echocardiographic time interval based index of myocardial performance. Milrinone increased biventricular function providing evidence that the direct myocardial effect of milrinone may improve low cardiac output syndrome in infants following cardiac surgery. While this study found evidence of improved ventricular function, it is not clear what other catecholamines the patients were on and how they interacted with milrinone. Although there was no control group, this group commented that a historical group of patients with low cardiac output syndrome treated with catecholamine alone during the same postoperative period had progressive deterioration in biventricular function.

Bailey *et al* reported an 18% increase in the cardiac index (2.9±0.2 to 3.4±0.3 L/min/m^2, $p<0.05$) in 20 children aged 3 to 22 months who received milrinone after surgical repair of congenital cardiac defects.[16] This study did not include neonates. All of these studies addressed the role of milrinone in low cardiac output syndrome after cardiac surgery. Although one can not assume that the mechanism of cardiac stunning in hypoxia-reoxygenation is identical to that found after cardiopulmonary bypass, the two conditions have similar pathophysiological phenotypes.

Septic shock can cause poor cardiac contractility, ventricular dysfunction and changes in systemic vascular resistance. Milrinone has also been used in the pediatric population to provide cardiovascular support during septic shock. A double blind randomized cross over study of 12 patients aged 9 months to 15 years with nonhyperdynamic septic shock despite catecholamine administration were given IV milrinone (50 μg/kg bolus; 0.5 μg/kg/min infusion) or placebo.[28] Echocardiography showed milrinone increased biventricular work index, stroke volume index and cardiac output with a subsequent increase in oxygen delivery. Systemic and pulmonary vascular resistances decreased and PAP was reduced. There was no significant difference between placebo and milrinone with respect to MAP or heart rate. All patients were adequately volume resuscitated prior to the administration of milrinone. This study did not include neonatal patients and only monitored the effects of milrinone infusion for 4h. The small sample size does not allow for adverse event detection. Despite these faults and limited literature, a task force on pediatric septic shock has included milrinone into the treatment flow scheme for septic pediatric and neonatal patients.[29]

A recent study by Paradisis *et al*, has used milrinone to prevent a form of low cardiac output syndrome found in preterm infants < 29 weeks gestational age as they transition from birth to cardiovascular stability.[12] This study excluded all babies with evidence of perinatal asphyxia (umbilical artery base excess of > -12) or preexisting significant hypotension (MAP < 24 mmHg). Ten infants received 0.75 μg/kg/min IV milrinone for 3h followed by 0.2 μg/kg/min until 18 h of age. All infants received 15 mL/kg of normal saline prior to milrinone administration. Forty percent of infants, respectively, developed hypotension or low superior vena cava flow requiring additional inotrope support. Superior vena cava flow was adequately maintained in the milrinone group compared to only 39% in historical controls. This regimen achieved a mean serum milrinone level of 212 ng/mL in 3h. There was a significant increase in heart rate at 3-6h with an increase in MAP between 9-18h. These authors suggested the therapeutic serum milrinone level of 180-300 ng/mL based on pediatric, not exclusively neonatal, pharmacokinetic model to develop their dosing regimen. The lack of an experimental control group however, does not allow us to conclude if milrinone or normal physiologic adaptations over time contributed to changes in MAP or superior vena cava flow. The small sample size also precludes the investigation into possible side effects. No measurements of cardiac output, organ flow (with the exception of middle cerebral artery flow), or oxygen metabolism were documented. These are all methodological concerns that must be addressed before routine use of milrinone in a neonatal population.[30]

There is a growing clinical interest in milrinone's ability to improve ventricular dysfunction while decreasing pulmonary vascular resistance. Two case series reports have demonstrated a role for milrinone in the setting of unresponsive pulmonary

hypertension, in term and growth restricted preterm neonates.[31,32] With milrinone administration, none of the neonates manifested hypotension but did show a clinical drop in pulmonary vascular resistance manifested by improved oxygenation and survival. Bassler *et al* used milrinone in combination with inhaled nitric oxide and proposed that the vasodilation of pulmonary vascular smooth muscle mediated by nitric oxide was enhanced by milrinone's ability to inhibit PDE, interfering with the breakdown of cAMP and cyclic guanosine monophosphate.[31] Danhaive *et al* suggested that milrinone's ability to increase right ventricular output and decrease pulmonary vascular resistance might help alleviate intrapulmonary shunting and decreased ventricular output associated with persistent pulmonary hypertension of the newborn.[32]

Selected Adult Studies

An adult study compared short term infusions of dobutamine, nitroprusside and milrinone in adults with severe heart failure.[33] Inotropic response to milrinone was preserved but attenuated with dobutamine. Milrinone and nitroprusside decreased pulmonary wedge pressure much more than dobutamine. Myocardial oxygen consumption increased with dobutamine but not with milrinone or nitroprusside.

Another study compared dopamine plus milrinone versus dopamine plus dobutamine for adult patients in shock.[34] Dopamine plus milrinone was advantageous with respect to preload and afterload reduction while preserving myocardial oxygen demand, but the authors warned against theoretical declines in arterial pressure causing end organ damage.

Long term oral milrinone therapy in adults with severe heart failure has been associated with a 53% increase in relative risk of mortality in the large multicenter Prospective Milrinone Survival Evaluation (PROMISE) trial.[35] Short term intravenous milrinone therapy used to support temporarily stunned or dysfunction myocardium has been well tolerated in adult and pediatric populations.[8]

Animal and Ex Vivo Studies of Phosphodiesterase Inhibitors During Normoxia

Neonatal animal studies of amrinone have demonstrated a variety of effect on cardiac tissue of fetus and newborns. Using neonatal rat myocardial cell preparations as well as cat and rabbit papillary muscle, Katz *et al* showed no positive inotropic effects of amrinone.[36] These studies were done with amrinone at doses exceeding therapeutic range and were done ex vivo; thus, can not be directly translated to an intact neonatal model of milrinone. In adult patients, milrinone has 10-75 times more positive inotropic potency than amrinone with a higher therapeutic index, less side effects, and a shorter half life.[11] Minimal or even negative inotropic effect at birth were found with "low-dose" milrinone (30-100 μg/mL) in mammalian muscle and piglet hearts.[37,38] However, this negative inotropy quickly changed into a positive inotropic effect within days of birth and at higher doses.[39]

Echocardiographic studies of supertherapeutic single dose of milrinone (1000 μg/kg) in 3 healthy adult dogs have shown mild hemorrhages in the subendocardium and myocardium of the left ventricle, thought to be secondary to increased ventricular workload.[40] While this study serves as a warning to monitor milrinone in the pediatric population, its applicability is questionable given the large dose (13 times of the highest

recommended loading dose in a neonate), the small number of animals, the lack of blinded investigators or control animals, and the use of healthy adult animals.

Isolated stomach wall of the guinea pig incubated with milrinone showed no adverse effect on ion transport of the tissue at milrinone levels known to increase ventricular function.[41]

Studies of Milrinone During Hypoxia

Animal studies to investigate the effects of milrinone during hypoxic injury have been performed. A study of ventilated dogs with hypoxia induced metabolic acidemia demonstrated that severe acidosis (pH <7.0), but not mild acidosis (pH <7.2) altered the ability of milrinone to increase left ventricular function and pulmonary blood flow. An accompanying in vitro study of ventricular preparations showed that severe acidosis and decreased milrinone effectiveness coincided with decreased intracellular cAMP levels.[42]

Studies with isolated cat papillary muscle found that milrinone was less active with decreased contractility when the sarcoplasmic reticulum was overloaded with calcium as during reoxygenation or in an immature sarcoplasmic reticulum.[43]

Milrinone has been used to treat hypoxic adult dogs in an intact in vivo experimental model. Milrinone increased cerebral and renal blood flow while decreasing pulmonary pressure and vascular resistance.[44,45] Mild to moderate decreases in MAP were found with no effect on cardiac output. No reoxygenation of the hypoxic dogs occurred. Single doses as high as 250 μg/kg were used which might have contributed to hypotension.

In lambs, milrinone has been used to treat hypoxia induced pulmonary hypertension and myocardial depression. Milrinone decreased PAP, decreased systemic vascular resistance without hypotension, and increased left ventricular function.[6]

All of these studies investigated the use of milrinone during a hypoxic state. Clinically, neonates are resuscitated and treated in a reoxygenated state as opposed to during hypoxia. The response of the reperfused and reoxygenated myocardium is different than that of hypoxic myocardium and may be complicated by the presence of oxygen free radical related injury.

Milrinone and Inflammation

Tissue reperfusion injury can lead to increased cytokines and tumor necrosis factor alpha resulting in cardiomyocyte dysfunction and low cardiac output syndrome. In addition, during the hypoxia-reoxygenation process there is an eventual depletion of high energy molecules such as (adenosine triphosphate and cAMP) that are used to fuel cell metabolism and function. There is evidence that cardiomyocytes have a decreased response to catecholamines in the presence of the combination of decreased cAMP and tumour necrosis factor alpha.[46] As well as increasing cAMP levels, literature suggests that milrinone may inhibit pro-inflammatory cytokine formation, and may therefore counter some of the damage caused by reoxygenation and reperfusion injury.[47] Milrinone has been shown to inhibit cAMP degradation and consume less oxygen while improving cardiac dysfunction.[1]

CONCLUSION

The literature on neonatal milrinone use is sparse with a few studies focusing on treating low cardiac output after cardiac surgery or in prematurity. Several case reports exist for the use of milrinone in neonates with unresponsive pulmonary hypertension. Postoperative management concerns for pediatric cardiac patients are similar to those for asphyxiated term neonates including improving cardiac contractility and blood pressure, decreasing diastolic dysfunction and afterload, improving pulmonary hypertension, and ameliorating oxygen free radical damage.

Currently, there are no clinical studies or animal models that have investigated the cardiovascular and hemodynamic effects of milrinone in resuscitated neonates after hypoxia. A greater understanding of milrinone's systemic and regional hemodynamic effects in a setting of neonatal hypoxia-reoxygenation could be garnered from an appropriate experimental model using neonatal animals.

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CHAPTER 5

The Experimental Model: Acute Hypoxia and Reoxygenation in Instrumented Newborn Piglets

EXPERIMENTAL DESIGN

Piglets as a Model of Neonatal Hypoxia-Reoxygenation

Newborn piglets are commonly used for perinatal research due to their anatomic and physiologic similarity to human neonates.[1]

In a literature review of animal models of neonatal hypoxia leading to encephalopathy, 26% of studies were in rodents, followed by piglets (23%), and fetal and newborn lambs (22%).[2] Piglets, newborn lamb and dogs were most often used to study acute metabolic changes during hypoxia.

Although piglets are a polytocous species, they are relatively mature at the time of birth with maturity comparable to the human neonate.[3] Piglet and human neonates have a similar body composition as well as distribution and size of blood vessels and pulmonary vasculature with comparable blood pressure, heart rate, cardiac output and regional blood flow distribution.[4-6] Swine baroreflex sensitivity increases from birth to six weeks of age, with higher blood pressures causing greater bradycardia in older piglets. These findings are consistent with the human postnatal maturation of the cardiovascular autonomic nervous system.[7] Piglet's intestinal physiology, anatomy, and motility are comparable to that of a human neonate.[8] The piglet brain at 1-4 days is comparable in size and maturation to the neonatal brain; however, angiography studies of piglet cerebral circulation have also demonstrated that the collateral communication between the two hemispheres, particularly between extra- and intracerebral arteries and vertebral and carotid arteries are very well developed in this animal comparable to that of the human neonate.[2,9] In contrast, the internal carotid artery in sheep is underdeveloped.[2]

The resting blood pH, blood gas parameters, lactates, serum chemistries and hematologic parameters are similar between the species.[10]

The cardiovascular and biochemical responses to alveolar hypoxia in piglets have been found to resemble those of a human neonate.[11-13] Acute cerebral metabolic outcomes after asphyxia have been well established in the piglet model.[2] The piglet pulmonary vascular smooth muscle constricts in response to alveolar hypoxia and reacts to anesthesia in a manner similar to human neonates.[6] The mesenteric response to low cardiac output and acute intestinal hypoperfusion are similar between humans and piglets. Although dogs have been used to study neonatal intestinal pathophysiology, the dog intestine is far more prone to bacterial translocation than the piglet; thus, clouding the systemic alterations associated with hypoxia-reoxygenation. In the presence of asphyxia with acidosis, piglet kidneys demonstrate a response similar to human neonates with decreased renal blood flow secondary to increased vascular tone of the efferent and afferent glomerular arterioles.[14]

Despite physiologic similarities, piglets mature quickly in comparison to human neonates; thus, piglets ≤ 3 days old were used in this model to achieve the closest possible comparison to the human newborn.[2] Taken together, similarities in their cardiovascular, gastrointestinal, pulmonary and neurological systems support the argument to regard piglets an ideal neonatal model for asphyxia and reoxygenation. However, there are potential differences in drug metabolism, maturation and number of adrenergic receptors, and cardiovascular responses to various insults. Nonetheless, among the animal models used, the piglet model has been commonly used for the investigation of asphyxia, reoxygenation and cardiovascular medications.[15,16]

The Use of an Acute Intact Animal Model with Instrumentation

The study of systemic and pulmonary hemodynamic responses to asphyxia, reoxygenation, and inotrope support requires *in vivo* monitoring of an intact model. Hemodynamic responses to asphyxia-reoxygenation are multi-faceted with strong interplay between organ systems, adrenergic receptors, cardiovascular reflexes, blood flow redistribution, and oxygen/substrate metabolism.

Animal models of milrinone have been predominantly focused on its effects on a single organ.[17,18] However, the effects of a drug on one organ can be appreciated only when organ systems are allowed to act as a whole. Given the renal excretion of milrinone, the result of asphyxia on the kidneys may have significant influence on milrinone blood levels and the consequent effect on the animal as a whole.

Although some researchers view the acutely instrumented animals as unphysiologic, the stress of surgery may mimic the clinical stress of intubation, ventilation, central venous line placement and arterial catheterization that many asphyxiated neonates in the intensive care unit endure. Physiologic stress from surgery was minimized by limiting the surgery to less than 80 min. In addition, a period of 40 min followed surgery to allow hemodynamic stabilization (±10% variation) together with the inclusion of sham-operated controls to ensure effects seen during hypoxia-reoxygenation and drug administration could be analyzed in a valid manner. The surgery was performed by the same group of investigators throughout the duration of the study (Appendix 1, Figure A-1). Block randomization also ensured that the effects of the experimental environment over the 2 months needed to complete study were distributed evenly among the treatment groups.

The piglet's ability to tolerate anaesthesia, surgical instrumentation, and the associated stress make them an ideal model for an instrumented *in vivo* model of perinatal stress.[19]

The Use of Anesthesia

Like the human neonate, piglets are physiologically sensitive animals. Unanaesthetized piglets do not withstand stress or asphyxiating insults well and thus most acute asphyxia-reoxygenation experiments with piglets must occur under sedation.[20-22] Fisher's work with short term hypoxia in unanaesthetized lambs demonstrated similar results with respect to myocardial blood flow to those studies with anesthetized lambs under similar conditions.[23,24] Consequently our experimental model includes the humane induction of anesthesia with halothane. Pancuronium was to induce a degree of muscle relaxation to coordinate ventilator support during hypoxia-reoxygenation. Under no circumstances were the animals paralyzed for behaviours interpretable as pain.

Concern exists that halothane may alter cardiopulmonary dynamics. Halothane can prevent an increase in lung resistance and decrease in compliance during ventilation but has been criticized for its possible cardiovascular side effects.[25] In newborn piglets, 1% halothane can decrease cardiac output and contractility, as well as resting heart rate and blood pressure.[26,27] Preload, afterload, as well as levels of cardiac adenosine triphosphate, creatine phosphate, and glycogen are unchanged with halothane.[26,28] Studies of ventilated dogs showed that sevofluorane had more untoward cardiovascular effects than halothane, while isoflurane reduced blood pressure and pulmonary resistance more than, and cardiac output less than, halothane did.[29] Heart rate and ventricular function

were decreased to a similar extent by both agents.[30] Nonetheless, in our study, the use of halothane was limited to a short duration and has been well established in the Neonatal Sciences Laboratory with a greater ease of delivery and predictability.[21,22]

There are also concerns that some piglets are predisposed to malignant hyperthermia with halothane. Piglets less than 8 wk of age do not usually develop characteristic hyperthermia and limb muscle rigidity in response to a brief exposure of halothane (5 min of 3%). The malignant hyperthermia defect is apparently partially masked in newborn piglets and usually expressed fully in older pigs.[31]

Due to the hemodynamic concerns surrounding halothane and its possible contamination of cardiovascular results in the piglet asphyxia-reoxygenation model, halothane exposure was brief (<20 min) at low concentrations (2%). The halothane was then discontinued and followed by the maintenance of analgesia and sedation with fentanyl and midazalom, respectively. This combination of medications can be used in the neonatal intensive care unit for severely asphyxiated neonates.

Analgesia and Sedation

Fentanyl is an opiate commonly used in neonates and can be associated with bradypnea and bradycardia at high doses. Rajan *et al* studied 6 healthy piglets who were given a loading dose of fentanyl at 30 μg/kg intravenously over 15 min followed by a continuous infusion at 10 μg/kg/h for 6h.[32] At this dose, fentanyl caused a transient increase in respiratory rate at 2h and increase in heart rate at 30 min and 6h. Fentanyl may produce cerebral vasoconstriction.[33] Our study used doses of fentanyl between 5-15 μg/kg/h and was titrated to the lowest doses required for adequate pain and sedation.

Midazolam, a sedative and anxiolytic is used to augment the effects of fentanyl such that lower doses of fentanyl can be used, avoiding the side effects of fentanyl such as hypotension or bradycardia that can be found at higher doses.

Our protocol also involves the optional administration of acepromazine for additional sedation and hemodynamic stability during instrumentation. Acepromazine maleate is a phenothiazine derivative used as an animal tranquilizer. Acepromazine has α_1 adrenergic blocking properties and can decrease vasomotor tone and dilate blood vessels. Heart and respiratory rate and thermoregulatory abilities can decrease, therefore, this drug was only given during instrumentation and in limited quantity to ensure that its effects did not contaminate those of hypoxia-reoxygenation.

Model of Hypoxia-Reoxygenation

This study induced normocapnic alveolar hypoxia (P_aO_2 30-40 mmHg) for 2h followed by reoxygenation with 100% oxygen (1h) then 21% oxygen for the remainder of the experiment (3h). Clinical experience in our neonatal intensive care unit has shown, on average, the time from the detection of fetal distress (heart rate abnormalities, decreased movements) to Caesarian section delivery was 130 min (personal communication with Dr. P-Y Cheung). Other neonates may be delivered emergently (with placental abruption) in less than 1h. However, the time until optimal resuscitation may be delayed. While it is recognized that hypoxia time and etiology can vary greatly between asphyxiated neonates, we believe that the 2h duration of hypoxia used in this protocol reflects the clinical scenario despite the absence of increased carbon dioxide. Two hours of normocapneic alveolar hypoxia in this model produces a pH $\approx$ 7.0,

neurological manifestations (hypotonia, coma) and multiorgan dysfunction (low cardiac output, hypotension, pulmonary hypertension) compatible with the definition of asphyxia by the American Academy of Pediatrics and American College of Obstetricians and Gynecologists.[21,22,34]

Current recommendations on the indications and percent of oxygen used for the resuscitation of asphyxiated term neonates are in flux. Recent neonatal resuscitation guidelines of the American Heart Association/American Academy of Pediatrics (Neonatal Resuscitation Program) still recommend the initial use of 100% oxygen for this population followed by the gradual reduction to 21% oxygen as the infant becomes more stable.[35] The 1h duration of 100% oxygen may be relatively long at a tertiary centre but is not uncommon for resuscitations that occur in a distant region that then requires medical transportation to the designated neonatal intensive care unit.

The Use of Transonic Flow Probes

Transonic Systems' ultrasonic transit-time technology has been used in animal research studies of volume flow measurement for more than two decades and validated in many applications with references in over 3,000 publications.[36,37]

Using wide-beam illumination, transducers pass ultrasonic signals back and forth, alternately intersecting the flowing liquid in the upstream and downstream direction. The speed of the ultrasound is affected by the flow of liquid. The Flowmeter derives an accurate measure of the 'transit-time' it took the wave of ultrasound to travel from one transducer to the other and produces a measure of volume flow[37] (Appendix 1, Figure A-2). There is a reported variability of ±10% that we attempted to minimize through

consistency of probe alignment, anatomic placement, and surgical technique (Appendix 1, Figures A-2 to A-6). Regular calibration of the flow probes was performed.

Prior to the introduction of ultrasonic flow probes, most studies of fetal and neonatal blood flow had been done with radioactive labeled microspheres. Microspheres allow for global and regional measurement of flows but only at specified times.[38] *In vivo* ultrasonic flow probes allow a continuous and dynamic monitoring of blood flow, thus the technique provides temporal measurement through physiologic conditions and pathophysiological events. Furthermore, some microsphere protocols do not ligate the ductus arteriosus and withdraw reference samples from the descending aorta, allowing a hypothetical escape of microspheres into the pulmonary circulation. If an experimental drug or procedure influences shunting through the ductus arteriosus, an artificially decreased cardiac output may be estimated due to loss of microsphere into the pulmonary circulation. By placing a transonic probe around the base of the main pulmonary artery with concurrent ligation the ductus arteriosus, an accurate measure of cardiac output can be achieved.

Carotid Blood Flow as an Estimation of Cerebral Blood Flow

The use of common carotid artery blood flow to estimate cerebral blood flow has been studied. Meadow *et al* evaluated the common carotid artery blood flow measured by Transonic flow probes and the cerebral blood flow by microsphere technique in piglets.[39] This study found that unilateral common carotid artery blood flow correlated with unilateral cerebral blood flow over a wide range of hypoxia but overestimated the cerebral blood flow by as much 68%. However, the dynamic nature of the flow probe

allowed significant correlation with increases and decreases in flow (slope of correlation 1.06 ± 0.15). This finding is corroborated by studies in lambs that showed a strong linear correlation with percent change in carotid blood flow and cerebral blood changes as opposed to only a modest correlation between absolute flows.[38,40,41] In contrast, sheep have an underdeveloped internal carotid artery that is absent in adult sheep while the anatomy of the piglet carotid artery is similar to that of a human neonate. Left and right cortical blood flows measured by microsphere technique were not significantly different indicating that the unilateral carotid placement of the probe and the surgical instrumentation did not alter the pattern of cerebral blood flow.[40] Alternatively, Doppler ultrasound velocity measurements through a patent fontanel are a non-invasive, clinically accessible method for assessing cerebral circulation. Piglets do not have an accessible patent fontanel. Inter-operator variability with respect to angle of insonication and technique can further lead to large variability between Doppler ultrasound velocity measurements and cerebral blood flow.[42]

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CHAPTER 6

Hemodynamic Effects of Milrinone in the Reoxygenation of Asphyxiated Newborn Piglets

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A version of this chapter has been submitted for publication to American Journal of Respiratory and Critical Care Medicine. Joynt CA, Bigam D, Charrois G, Jewell LD, Cheung PY.

High Performance Liquid Chromatography and sample preparation of plasma for milrinone level determination was performed by Ken Strynadka and John Ussher. Plasma lactate assays were performed by Corinne Tymafichuk.

INTRODUCTION

Neonatal asphyxia often leads to a low cardiac output state and left ventricular myocardial dysfunction. This may put sick neonates at risk for decreased blood flow and oxygen delivery to vital organs including the brain, leading to significant morbidity and mortality.[1] Following asphyxia, neonates may develop reduced myocardial contractility and passive cardiac dilatation often in conjunction with cardiac stunning, systemic hypotension and pulmonary hypertension (PHT). [1-4]

Current treatments for hypotension in asphyxiated neonates include catecholamines such as dopamine, epinephrine, and dobutamine. Dopamine and epinephrine, at high doses, can increase heart rate and vascular resistance at the expense of increased oxygen consumption, fluctuating blood pressures and aggravating PHT.[5] Dobutamine can cause increased oxygen consumption, tachycardia and arrhythmias despite its inotropic effect in asphyxiated neonates.[6,7] However, some asphyxiated neonates have low systemic blood flow without severe hypotension due to poor myocardial contractility.[2] In these patients inotropic stimulation to augment myofibril contractility may be more appropriate to combat myocardial stunning after reoxygenation or reperfusion.[8]

Milrinone is a specific phosphodiesterase (PDE)-III inhibitor that increases myocardial contractility by elevating the intracellular cyclic adenosine monophosphate (cAMP) concentration through the specific inhibition of PDE-III on cAMP degradation.[9] It can increase cardiac stroke volume thus cardiac output and improve diastolic dysfunction.[10] Vasodilatory effects of milrinone can alleviate PHT but may also decrease systemic arterial pressure.[5,11,12] Compared with currently used inotropes, PDE-III

inhibitors may increase cardiac output without concomitant increase in myocardial oxygen consumption.[12,13]

Few studies specifically address the effects of milrinone in neonates. This literature contains neonatal patients who had low cardiac output after cardiac surgery, premature infants at risk for hypotension, or those with unresponsive PHT.[12,14-16] In these studies, doses of milrinone used and hemodynamic parameters measured are variable. Management strategies for patients after cardiac surgery or PHT are similar to those for asphyxiated term neonates including improving contractility, decreasing diastolic dysfunction, reducing afterload and improving pulmonary vasculature hemodynamics.[17] There is no literature on the use of milrinone in asphyxiated neonates regarding the systemic and regional hemodynamic effects.

Using a swine model of neonatal hypoxia and reoxygenation (H-R), we primarily examined the dose-response effects of milrinone on the cardiac output and systemic blood pressure. The secondary objectives of the current study included the effects of milrinone on the pulmonary and cerebral circulations as well as oxygen transport. We hypothesized that milrinone would dose-dependently decrease H-R induced cardiac dysfunction and increase blood flow to the brain without aggravating PHT and with minimal effect on systemic blood pressure in hypoxia- reoxygenated piglets.

METHODS

Mixed breed piglets 1-3 days of age weighing 1.5-2.3 kg were obtained from a local farm on the day of experimentation. The experimental protocol was approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee.

Anesthesia

The piglets were initially anesthetized with 5% halothane and maintained at 2-3%. Inhalational anesthesia was discontinued upon initiation of mechanical ventilation via a tracheostomy. Anesthesia, analgesia, and paralysis were maintained with intravenous midazolam (0.1-0.2 mg/kg/h), fentanyl (5-15 μg/kg/h) and pancuronium (0.05-0.1 mg/kg/h) respectively, with up to two boluses of acepromazine (0.25 mg/kg). Inspired oxygen concentration (FiO_2) was measured by an Ohmeda 5100 oxygen monitor (Ohmeda Medical, Laurel, MD) and maintained at 0.21-0.24 to keep oxygen saturations between 90-100%. Percutaneous oxygen saturation was measured by continuous pulse oximetry (Nellcor, Hayward, CA). Blood pressure and heart rate were monitored with a Hewlett Packard 78833B monitor (Hewlett Packard Co., Palo Alto, CA). An intravenous 10% dextrose infusion at 10 mL/kg/h maintained glucose levels and hydration. An arterial line was maintained with an infusion of 0.9% normal saline at 4 mL/h. Body temperature was kept at 38.5-39.5°C using an overhead warmer and heating pad.

Surgical Procedure

A 5 F Argyle® double lumen catheter (Sherwood Medical Co., St Louis, MO) was inserted to the level of the right atrium via the femoral vein to measure central

venous pressure and to deliver fluids and medications. A 5 F Argyle® single lumen catheter was inserted into the right femoral artery, advanced into the infrarenal aorta and connected to a pressure transducer to continuously measure mean arterial pressure (MAP). Endotracheal intubation via a tracheostomy allowed pressure control assisted mechanical ventilation by an infant ventilator (model IV-100, Sechrist Industries Inc., Anaheim, CA) at a rate of 18-20 breaths/min and pressure of 19/4 cm H_2O.

Through a neck incision, the left common carotid artery was isolated with minimal dissection and encircled by a 2-mm Transonic® flow probe (2SS Transonic Systems Inc, Ithica, NY) to measure blood flow. Through a left anterior thoracotomy, the main pulmonary artery was exposed and cannulated with a 20-G Arrow® catheter (Arrow International, Reading , PA) connected to a pressure transducer to measure pulmonary arterial pressure (PAP). A 6-mm Transonic® flow probe (6SB) was placed to monitor the blood flow as a surrogate of cardiac output. The ductus arteriosus was ligated.

Incisions were covered and kept moist to minimize evaporative losses. Transonic® flow probes were connected to a T206 2-channel small animal flow meter. Transonic® flow probe and pressure transducer outputs were recorded and digitized by a DT2801-A analogue to digital converter board (Data Translation, Ontario, Canada) in a Dell 425E computer equipped with custom Asyst programming software.

Hypoxia-Reoxygenation and Treatment Procedure

Piglets were block randomized to 4 groups (n=7/group) which underwent H-R. A sham operated group of piglets (n=4) underwent instrumentation and stabilization without H-R or medication delivery.

After instrumentation, the animals underwent a period of stabilization for 40 min. Stability was defined as a hemodynamic measurement change ±<10% over 20 min, pH 7.35-7.45, P_aO_2 of 60-100 mmHg, and P_aCO_2 of 35-45 mmHg. Normocapneic alveolar hypoxia was initiated with the inhalation of an oxygen/nitrogen gas mixture to provide a FiO_2 of 0.10-0.15 and obtain a P_aO_2 of 20-40 mmHg for 2h. Previous studies have determined that in this piglet model, this degree of hypoxemia will produce clinical asphyxia with severe metabolic acidosis, systemic hypotension, a decrease in cardiac index (CI) to 40% of normoxic baseline and PHT.[7,18] Piglets were then reoxygenated with 100% oxygen for 1h and a further 3h of 21% oxygen. All piglets received a 10 mL/kg normal saline bolus 30 min prior to medication delivery. At 2h of reoxygenation, piglets received an intravenous infusion of placebo (normal saline) or high-dose milrinone (75 μg/kg loading, 0.75 μg/kg/min infusion), mid-dose milrinone (50 μg/kg, 0.75 μg/kg/min), or low-dose milrinone (25 μg/kg, 0.25 μg/kg/min) for 2h in a blinded fashion (Appendix 2).

Medication Preparation and Delivery

To maintain blinding, all doses of milrinone (Milrinone Lactate, Pharmaceutical Partners of Canada Inc., Richmond Hill, ON) and normal saline were reconstituted in a standard volume. The drug infusion was run at 6.7 mL/kg/h for 15 min (medication

loading bolus) followed by 1 mL/kg/h for the remainder of 2h. Medication infusions were mixed by a laboratory technician not involved in the experiment and identified by number only. All medications were clear and odorless and mixed just prior to use.

Hemodynamic and Oxygen Transport Measurements

Systemic (heart rate, pulmonary artery blood flow, MAP, PAP, central venous pressure) and carotid hemodynamic parameters (left common carotid artery blood flow), blood gases and co-oximetry were recorded at post surgical stabilization (0 min), every 30 min during hypoxia, at 0, 10, 30, 60, and 120 min of reoxygenation, and every 30 min following medication or placebo delivery (Appendix 3). Variables were calculated as a mean over 2 min at specified time points. Highest and lowest hemodynamic parameters were recorded for the duration of medication boluses at the beginning of medication administration. Hemodynamic variables were calculated as shown in Appendix 4.

At specified time points, simultaneous arterial and venous blood samples were taken for blood gas analysis, hemoglobin measurement, and co-oximetry by ABL500 (Radiometer, Copenhagen, Denmark) and OSM3 Hemoximeter (Radiometer). Calculations for systemic oxygen transport and carotid oxygen delivery are listed in Appendix 4.

Arterial blood samples (1 mL) were taken at specified time points, centrifuged at 15,000 rpm for 10 min, and the supernatant collected and frozen at -80°C for subsequent lactate determination. Less than 5% of the piglet blood volume was collected as bloodwork.

Left Ventricle Myocardium Sampling

At the end of the study, piglets were euthanized with 100 mg/kg pentobarbital IV. Samples of left ventricle were snap frozen in liquid nitrogen and stored at -80°C while another sample was fixed in 10% formalin for subsequent biochemical and histologic analysis, respectively.

Lactate as a Marker of Anaerobic Metabolism

Plasma levels of lactate were determined using the enzyme-coupled NAD colorimetry method.[19] Plasma samples taken prior to and at the end of hypoxia and medication delivery were removed from storage at -80 °C. Samples (15 μL) were removed and diluted with ddH_2O (110 μL). Colormetric microplate assay was performed with the addition of glycylglycine buffer, pH 10, NAD, ddH_2O, glutamate-pyruvate transaminase, and lactate dehydrogenase. The absorbance was read at 340 nm using a microplate spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, CA). The lactate concentration was calculated from a standard equation (Appendix 5).

For left ventricle tissue lactate determination, frozen heart tissue (50 mg) was crushed at -80°C and then homogenized in 6% perchloric acid/0.5mM EGTA (50 μL) on ice. After centrifuging at 11,000 rpm for 2 min at 4°C, the supernatant was weighed and 5M potassium carbonate was added slowly in a ratio of 1 μL:10 μL of supernatant. Precipitation on ice for 30 min was followed by centrifuging at 11,000 rpm for 2 min (Appendix 6). The supernatant was then used in substitute of plasma in the lactate assay described above.

Histopathology

Left ventricle specimens preserved in formalin were prepared for histological assessment using hematoxylin and eosin staining. Two independent pathologists (Jewell LD, Charrois G) who were blinded to treatment group evaluated the specimens and pathologic injury was graded based on previously established criteria.[20]

Plasma Milrinone Levels

Plasma samples previously stored at -80°C were used in a high performance liquid chromatographic validated method for the determination of milrinone in plasma.[21] Samples (100 μL) were mixed with internal standard (amrinone 10 μg/mL), 0.15 mL of water and 0.35 mL of acetonitrile and centrifuged at 3,800g x 3 min. The supernatant was collected and 3 mL of 95:5 diethyl ether: methanol was added followed by centrifugation at 3,800g x 3 min again. The organic solvent layer was transferred and dried in vacuo, then reconstituted with 150 μL of the mobile phase (90:7:30 [25 mM KH_2PO_4: 3 mM sulfuric acid: 3.6 mM triethylamine]: methanol: acetonitrile). Samples were chromatographed on a C18 column at 21°C. Detection of milrinone and the internal standard was achieved by UV detection at 326 nm. Signals from the detector were collected and recorded. Standard of milrinone (0-2000 ng/mL) were used as standards. The mean r^2 for standard curves in human plasma is $0.999 \pm 7.9 \times 10^{-5}$. The limit of quantitation was 5 ng/mL.

Statistics

Using an $\alpha<0.05$ and $\beta<0.2$, a sample size of 7 piglets per treatment group was estimated to demonstrate a statistically significant difference in CI between groups based on our previous experience. Results are expressed as mean ± SEM. Hemodynamic variables were analyzed by 2-way ANOVA followed by 1-way RM ANOVA for differences within groups over time and 1-way ANOVA for differences between groups at a given time point. Biochemical variables were analyzed by 1-way ANOVA. If normality failed, ANOVA on ranks (Kruskal-Wallis) was performed. For post hoc testing, the Tukey or Dunnett's method was used where appropriate for pairwise comparisons. Pearson Moment correlation was used to determine the relationship between hemodynamic and milrinone levels. Analysis was performed using Sigma Stat (Version 2.0, Jandel Scientific, San Rafael, CA). Significance was defined as $p<0.05$.

RESULTS

The piglets were 2.0±0.2 days old, 28% female, with a weight of 1.86±0.04 kg. Mortality in this model was 11% (4 of 36 piglets). Four piglets died from complications secondary to surgery (2) and severe hypoxia (2). There were no significant differences among the groups with respect to age, weight, and mortality.

Stabilization

During stabilization, prior to the initiation of hypoxia, the normoxic baseline MAP was 70±2 mmHg with a heart rate of 193±6 beats/min and CI of 173±7 mL/kg/min. Pulmonary arterial pressure was 25±1 mmHg. The left common carotid flow index (CAFI) was 22±1 mL/kg/min. There were no significant differences in the hemodynamic variables among the groups.

Hypoxia

Two hours of normocapnic alveolar hypoxia was achieved with P_aO_2 34±1 mmHg and a metabolic acidosis of pH 7.04±0.04. During the experimental period, P_aCO_2 was maintained between 35-45 mmHg. Hypoxia reduced the CI to 71±4 mL/kg/min (41±1% of the normoxic baseline) with a decrease in MAP to 28±2 mmHg (41±2%) and a decrease in systemic vascular resistance index (SVRI) (76±5%). The stroke volume index was decreased to 41±3% of baseline. Hypoxia and metabolic acidosis resulted in PHT with PAP increased to 31±1 mmHg (128±6%) with an increase in pulmonary vascular resistance index (PVRI) of 315±16% of baseline. Compared to CI, CAFI was reduced to

a lesser extent to 14±1 mL/kg/min (66±5%). There were no significant differences in hemodynamic parameters among hypoxic groups.

Reoxygenation

Upon reoxygenation with 100% oxygen, hemodynamics immediately recovered to baseline as measured at 10 and 30 min. This recovery was not sustained, as the hemodynamic parameters gradually deteriorated over the next 90 min of reoxygenation. Despite normalization of oxygen saturation (>92%), P_aO_2 (61±1 mmHg), and pH (7.38±0.01) after 2h of reoxygenation, MAP fell to 67±3%, CI to 75±3% and CAFI to 79±3% of the respective normoxic baseline. Relative PHT continued with a PVRI at 142±5% of normoxia baseline compared to a relative hypotension with an accompanying decreased SVRI (85±5%) and diminished stroke volume index (67±3%). No significant differences in hemodynamic parameters were found among groups after 2h of reoxygenation prior to medication delivery.

After Milrinone or Placebo Infusion

Systemic Hemodynamics

When the loading dose of milrinone was given, there were no significant decreases or increases in MAP, PAP, heart rate or CI. No dysrhythmias were found with bolus milrinone.

As seen in Figure 6-1, all doses of milrinone increased CI after 2 h of infusion (vs. hypoxic controls with placebo infusion, $p<0.001$, ANOVA). A trend towards a milrinone dose-response was found with a greater effect of high-dose milrinone on CI than low and

mid-dose milrinone when CI is expressed as a percentage of pretreatment baseline (121±6% vs. 104±4% and 103±7% respectively) ($p=0.07$, $\beta= 0.6$). After 2h of treatment, the stroke volume index was increased with all doses of milrinone compared to placebo ($p=0.01$) (Figure 6-2). There was no significant difference in heart rate between milrinone groups and placebo after 2h of treatment (Figure 6-3).

Mean arterial pressure was maintained with milrinone as there was no significant difference between pretreatment and posttreatment MAP with milrinone (Figure 6-4). No significant difference in MAP was noted among groups during the 2h of treatment.

Systemic vascular resistance index was lower in the high and mid-dose milrinone treated groups compared to hypoxic controls ($p<0.05$). Low-dose milrinone showed a trend toward lower SVRI ($p<0.1$ vs. hypoxic controls). A trend towards a dose-response was found with high-dose milrinone lowering SVRI more than low-dose ($p<0.1$) (Table 6-1).

Pulmonary Hemodynamics

Pulmonary arterial pressure did not significantly differ among treatment groups and did not significantly increase over the pretreatment baseline (Figure 6-5).

After 2h of treatment, all milrinone groups demonstrated significant decreases in the PVRI (vs. hypoxic controls, $p<0.05$) (Table 6-1). However, a significant difference was found in the PVRI between the hypoxic control and mid-dose groups at the pretreatment baseline ($p=0.02$). Post treatment (2h) values of PVRI expressed as a percentage of pretreatment baseline, to compensate for the different pretreatment baselines, demonstrated a significant decrease in PVRI in all milrinone groups compared

to control ($p< 0.05$). There was a trend towards a dose-response effect on PVRI (high vs. low-dose milrinone, $p< 0.1$).

Carotid Hemodynamics

High-dose milrinone infusion increased CAFI significantly over the hypoxic control group ($p<0.05$) whereas mid-dose milrinone demonstrated a trend towards increased CAFI. ($p =0.09$, $\beta= 0.4$) (Figure 6-6). Low-dose milrinone had no significant effect on CAFI. Carotid vascular resistance was slightly decreased in the high-dose milrinone to 86±7% of pretreatment baseline compared to the hypoxic control group at 108±9% ($p<0.1$, $\beta=0.3$ and $p=0.02$, t-test).

Oxygen Transport

All milrinone groups had higher systemic oxygen delivery compared to controls ($p<0.05$) (Figure 6-7). Systemic oxygen consumption was increased in all milrinone groups ($p<0.05$), with no significant difference in systemic oxygen extraction compared to the hypoxic control group. Carotid artery oxygen delivery was significantly higher in high and mid-dose milrinone groups compared to hypoxic controls ($p<0.05$) (Figure 6-8). In the plasma lactate measurements, there was a significant difference in pretreatment baseline between control and high groups ($p<0.05$). Therefore, to examine if milrinone treatment had any effect on the plasma lactate levels, end of treatment levels are expressed as a percentage of pretreatment baseline. There was no significant difference in plasma lactate between milrinone and the hypoxic control groups after 2h of treatment (Figure 6-9).

Plasma Milrinone Levels

There is a significant difference in plasma milrinone levels with a dose response among milrinone treatment groups (Figure 6-10).

Plasma milrinone levels correlated significantly with measures of cardiac function as indicated by CI and stroke volume index ($r=0.6$ and 0.5, respectively, $p<0.01$) (Figure 6-11). Modest correlations were found between milrinone levels and CAFI ($r= 0.5$, $p<0.05$) and carotid oxygen delivery ($r= 0.5$, $p<0.05$). Significant negative correlations were also found between milrinone levels and SVRI, PVRI and carotid vascular resistance (all $r=-0.6$, $p<0.05$).

Cardiac Tissue Analysis

There was no significant difference in cardiac histopathology between groups. None of the myocardial tissue samples demonstrated prominent ischemic changes or overt necrosis (Figure 6-12). The left ventricle cardiac tissue harvested at the end of the study protocol did not show a significant difference in lactate levels (Figure 6-9).

Side Effects

Intestinal pneumatosis (1, low-dose), and seizures (1, control) were observed in the study.

TABLE 6-1: SYSTEMIC AND PULMONARY VASCULAR RESISTANCE INDEX

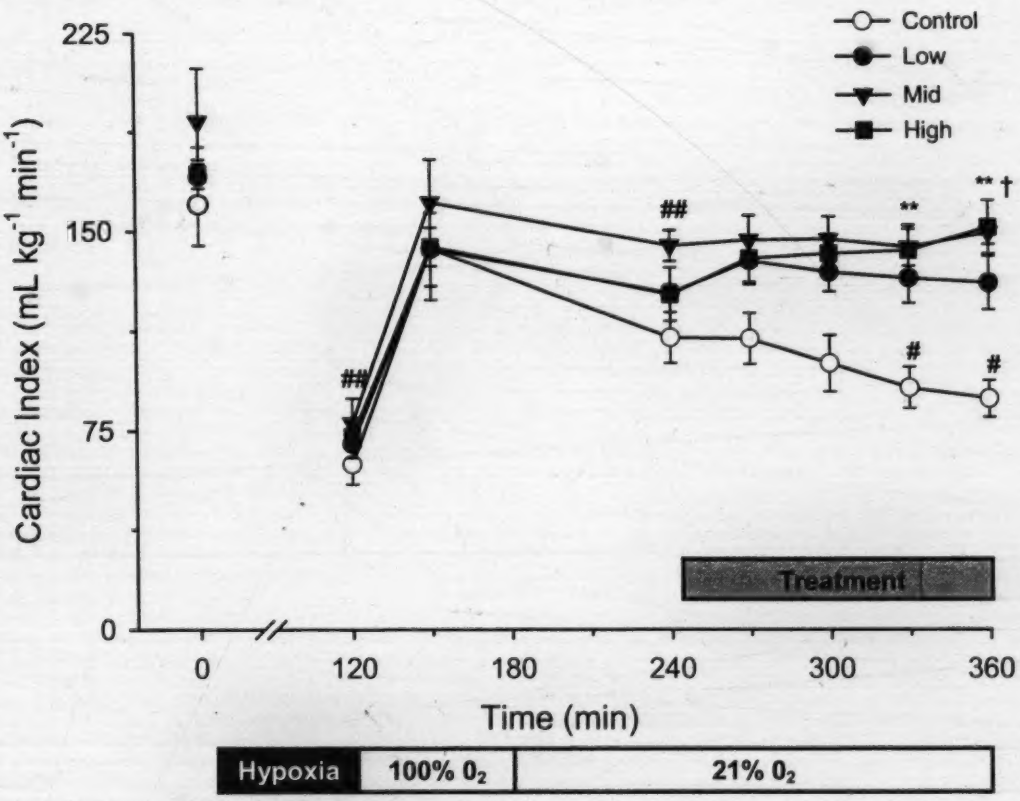
	Prehypoxia Baseline	*End of Hypoxia*	*Pretreatment Baseline*	*At 2h Medication (% pretreatment baseline)*
SVRI (mmHg mL^{-1} kg^{-1} min^{-1})				
Control	0.41±0.05	0.36±0.07	0.32±0.03#	0.37±0.04 (122±18%)
Low	0.41 0.04	0.31±0.03#	0.33±0.02#	0.32±0.02‡ (103±12)
Mid	0.34±0.04	0.23±0.03#	0.29±0.03#	0.27±0.01*# (94±3)
High	0.40±0.03	0.27±0.02#	0.29±0.02#	0.26±0.01*†# (90±6)
PVRI (mmHg mL^{-1} kg^{-1} min^{-1})				
Control	0.16±0.02	0.56±0.09#	0.25±0.03	0.37±0.03# (154±17)
Low	0.15±0.01	0.43±0.05#	0.21±0.01	0.24±0.02* (115±6)
Mid	0.12±0.01	0.39±0.04#	0.17±0.01*	0.20±0.02* (120±10)
High	0.15±0.01	0.44±0.04#	0.21±0.02	0.20±0.02*† (98±7)

* $p<0.05$ and ‡ $p<0.1$ vs. control

† $p<0.1$ vs. low milrinone group

# $p<0.05$ vs. normoxia baseline

FIGURE 6-1: CARDIAC INDEX



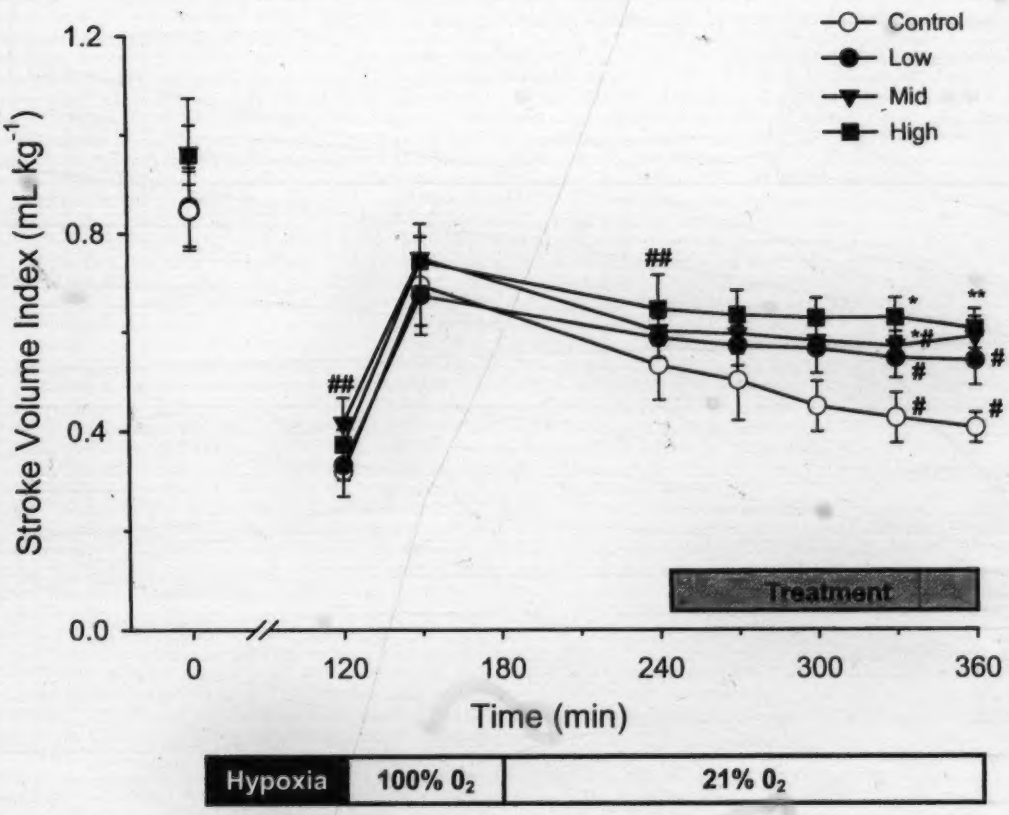
** p<0.05 all milrinone groups vs. control

† p=0.07 high vs. mid, low milrinone groups (121% vs. 105%, 103% of respective pretreatment baseline)

p<0.05 all groups vs. normoxic baseline

# p<0.05 individual group vs. normoxic baseline

FIGURE 6-2: STROKE VOLUME INDEX



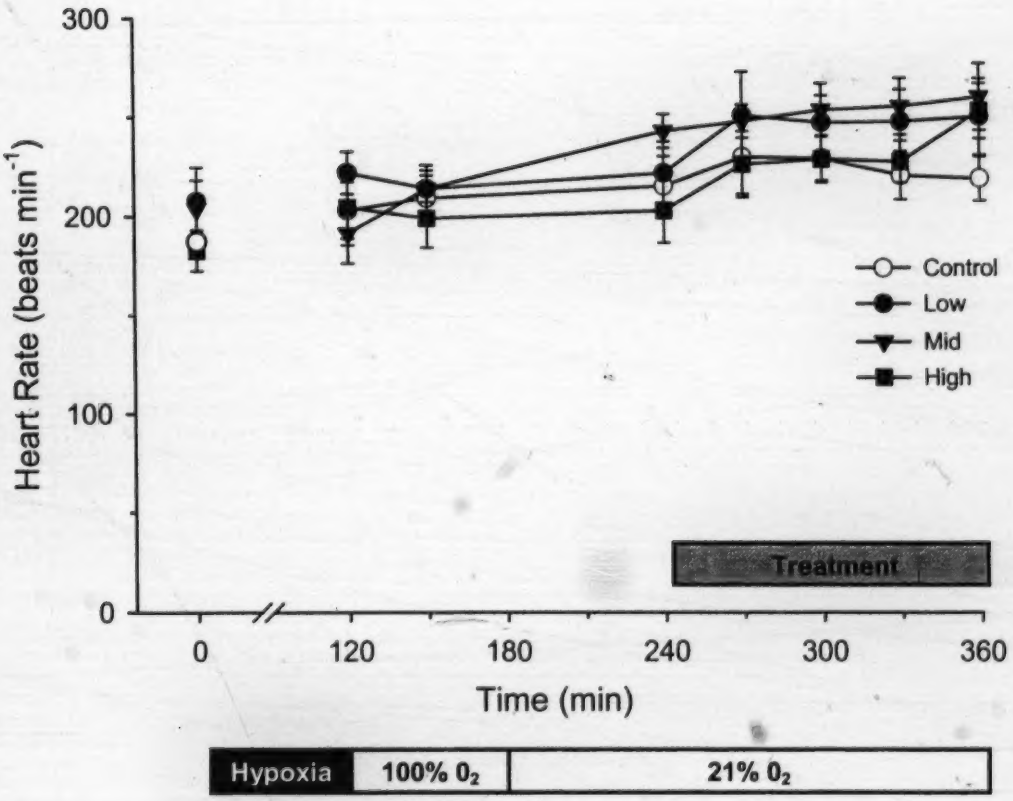
** p<0.05 all milrinone groups vs. control

* p<0.05 individual milrinone groups vs. control

p<0.05 all groups vs. normoxic baseline

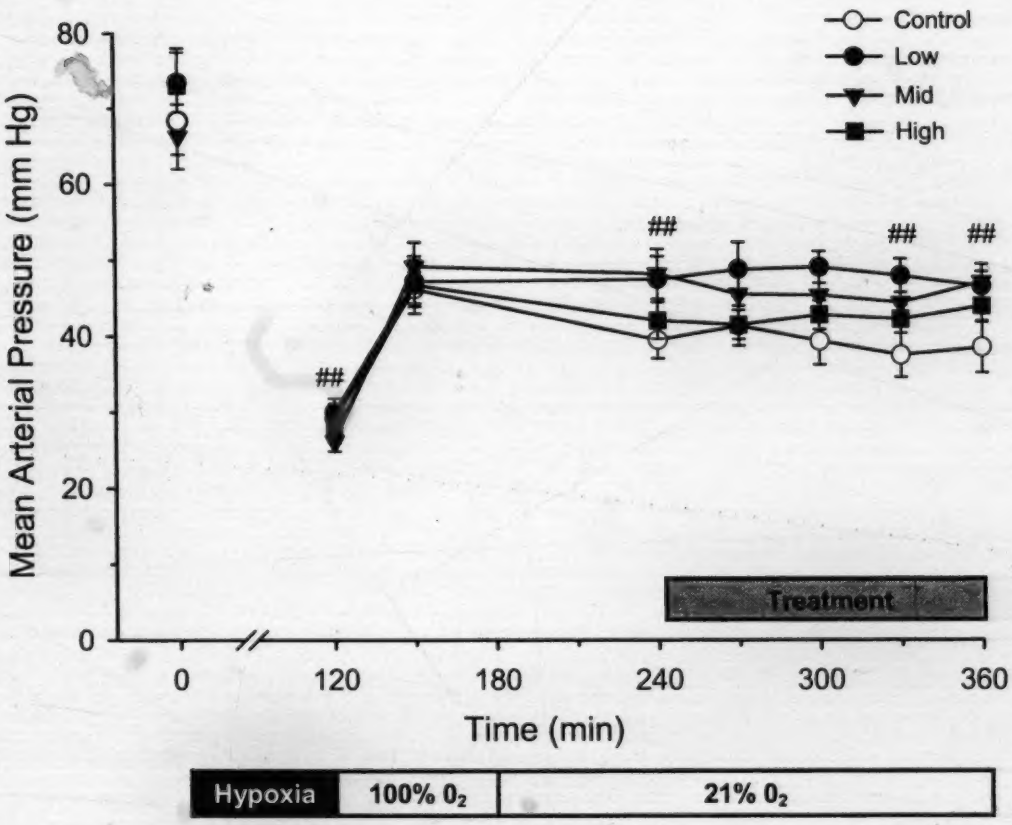
# p<0.05 individual group vs. normoxic baseline

FIGURE 6-3: HEART RATE



No significant difference was found among groups.

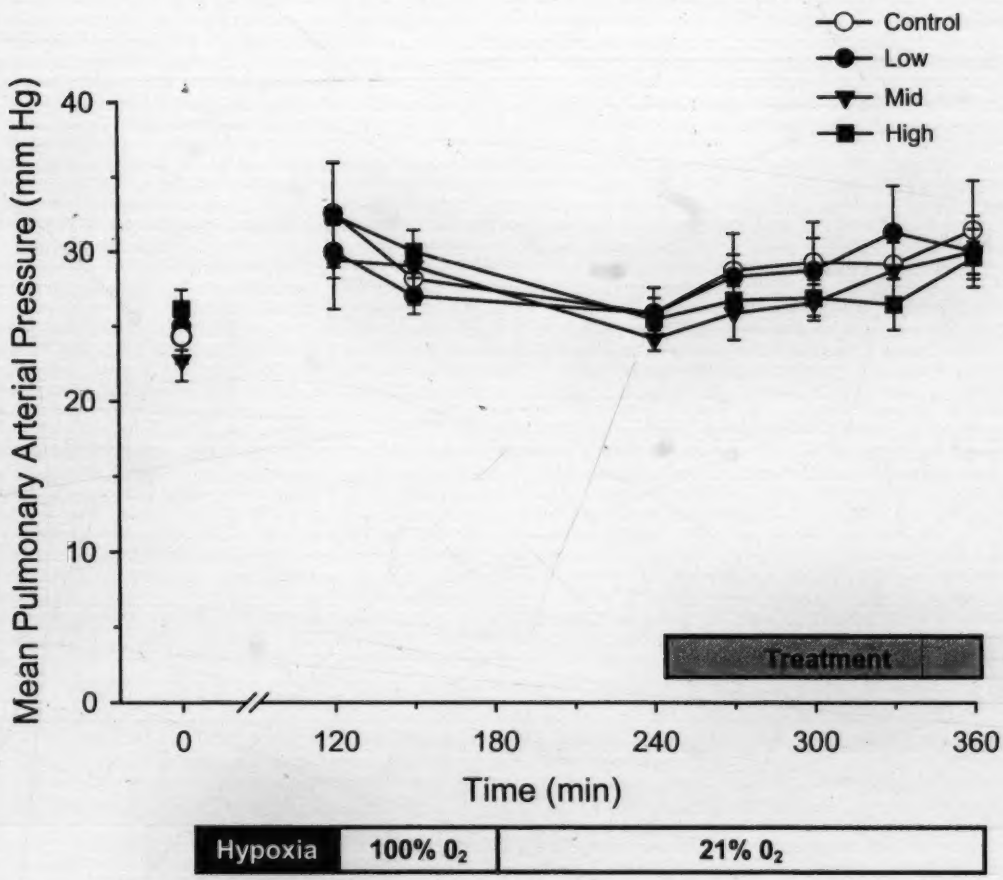
FIGURE 6-4: MEAN ARTERIAL PRESSURE



$p<0.05$ all groups vs. normoxic baseline

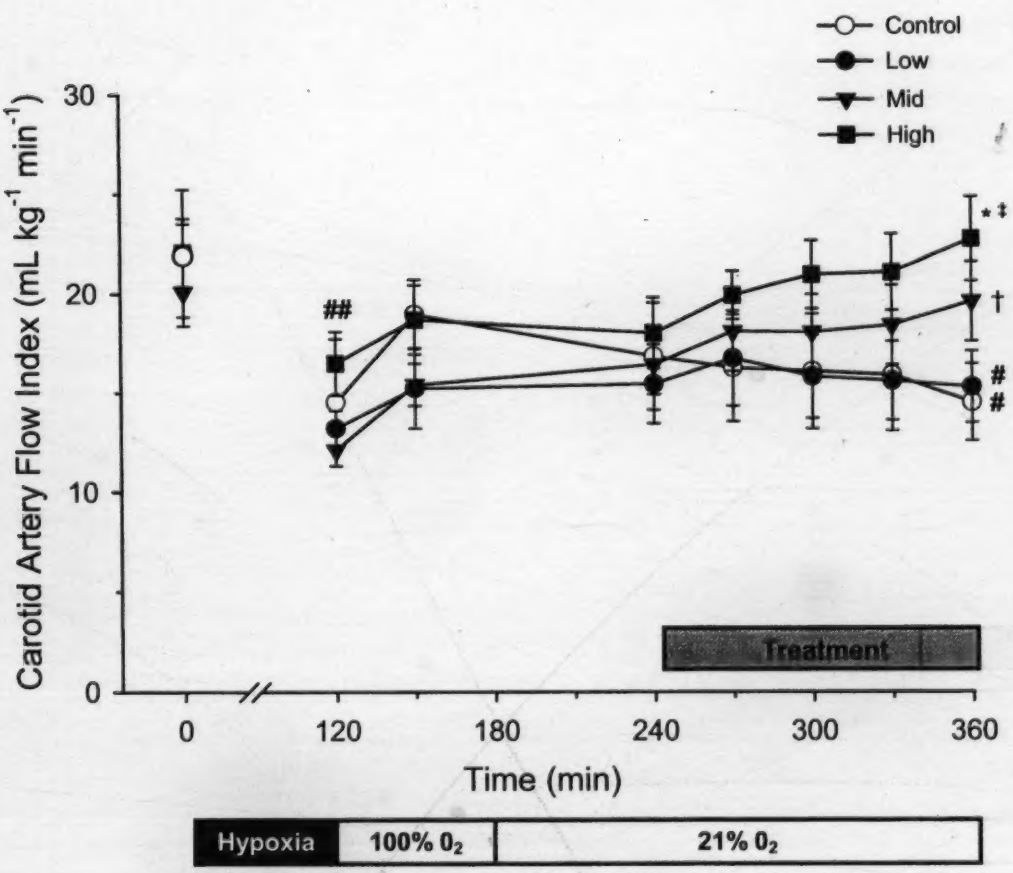
No significant difference was found between groups. The administration of milrinone maintained the mean arterial pressure without causing significant hypotension.

FIGURE 6- 5: PULMONARY ARTERIAL PRESSURE



No significant difference was found among groups.

FIGURE 6-6: CAROTID ARTERY FLOW INDEX



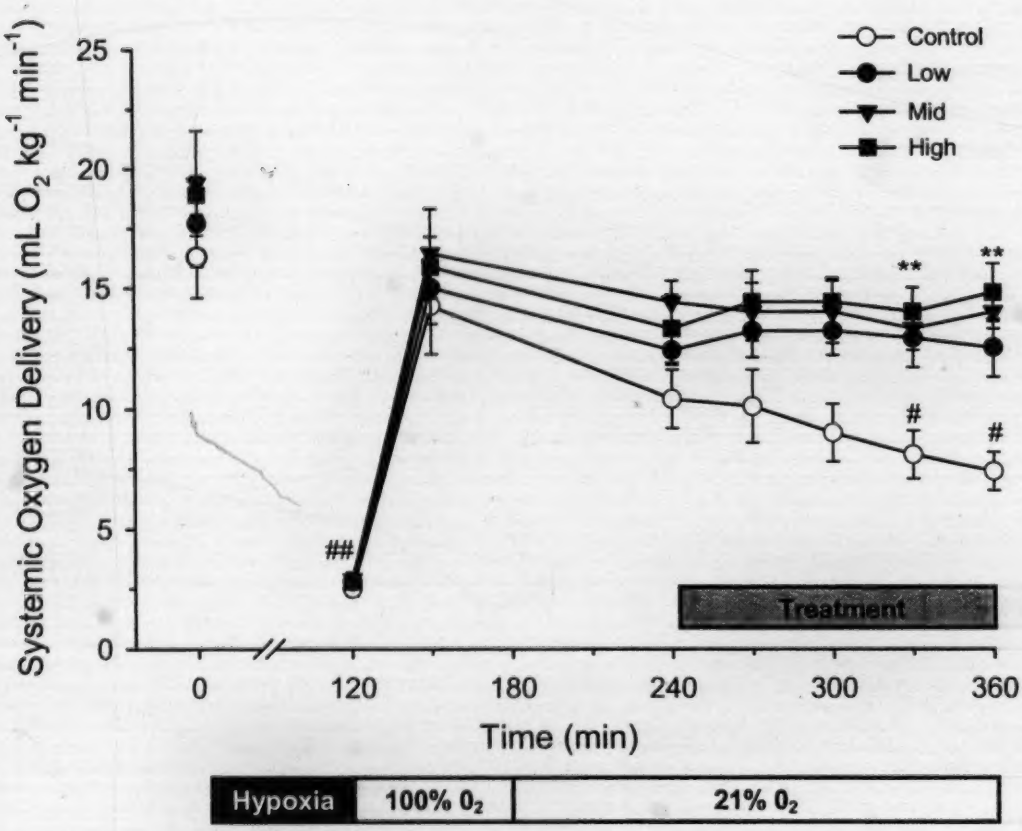
* $p<0.05$ and † $p<0.1$ individual milrinone group vs. control

‡ $p<0.05$ high vs. low-dose milrinone group

$p<0.05$ all groups vs. normoxic baseline

\# $p<0.05$ individual group vs. normoxic baseline

FIGURE 6-7: SYSTEMIC OXYGEN DELIVERY

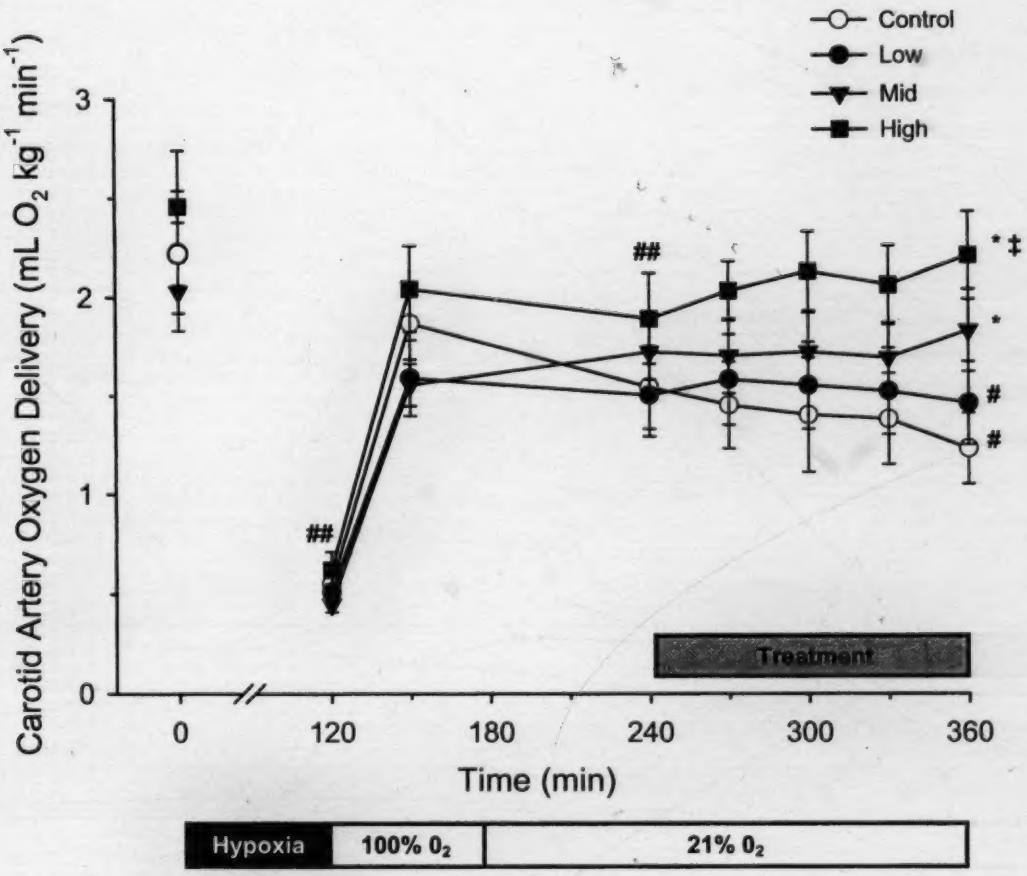


** $p<0.05$ all milrinone groups vs. control

$p<0.05$ all groups vs. normoxic baseline

\# $p<0.05$ individual group vs. normoxic baseline

FIGURE 6-8: CAROTID ARTERY OXYGEN DELIVERY



* p<0.05 individual milrinone group vs. control

‡ p<0.05 high vs. low-dose milrinone group

p<0.05 all groups vs. normoxic baseline

\# p< 0.05 individual group vs. normoxic baseline

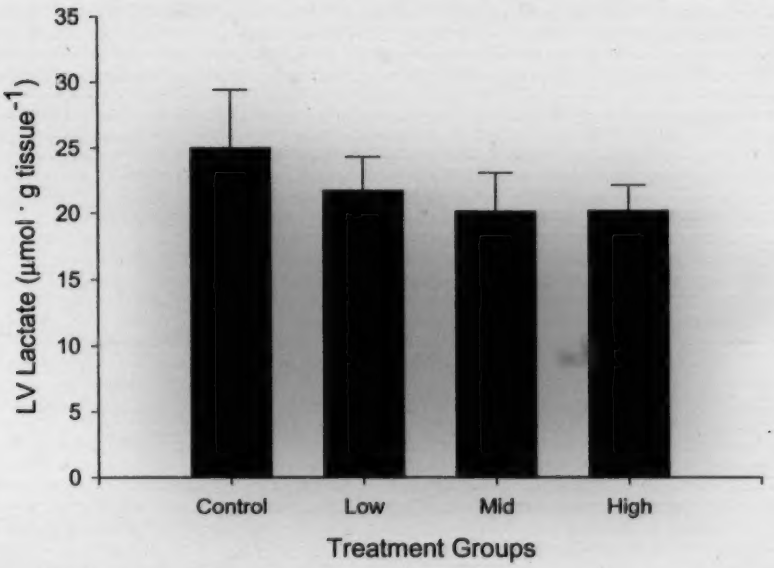
FIGURE 6-9: LACTATE MEASUREMENTS

(A) Plasma Lactate (mmol/L)

Treatment Group	*Prehypoxia Baseline*	*End of Hypoxia*	*Pretreatment Baseline*	*2h Treatment (% pretreatment)*
Control	4.4±0.5	16.0±0.8	7.0±0.2	4.7±1.1 (64±10%)
Low	3.9±0.3	15.4±0.6	5.2±0.5	3.0±0.4 (58±4)
Mid	3.7±0.3	14.5±1.4	5.4±0.8	2.7±0.3 (54±6)
High	4.2±0.4	14.8±0.7	4.0±0.4*	2.8±0.7 (72±16)

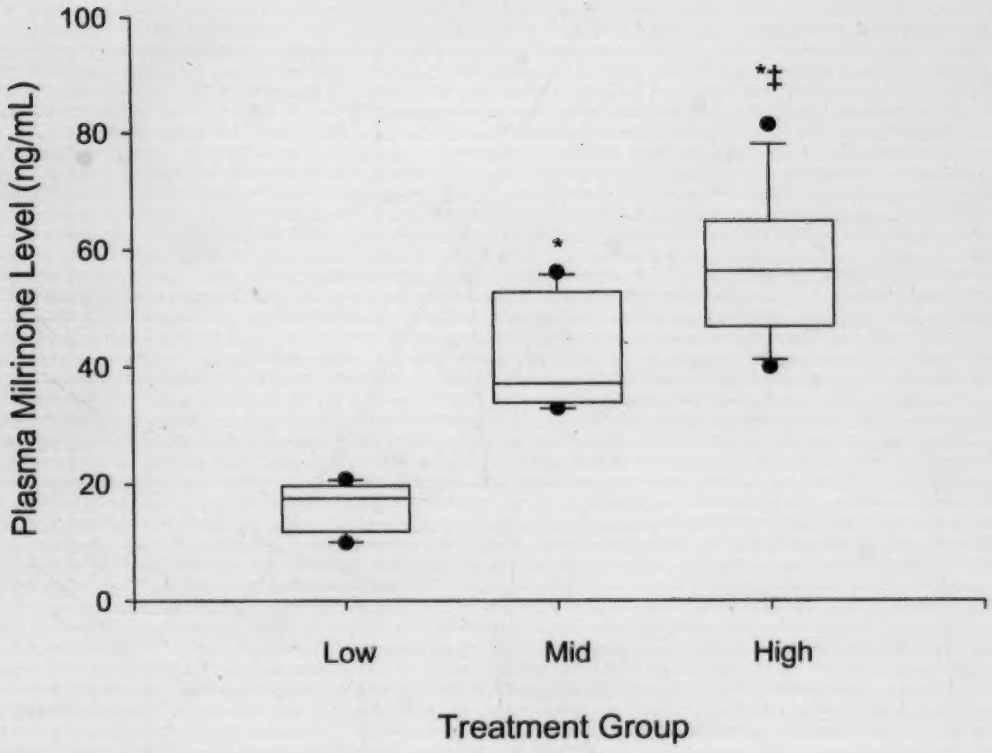
* $p<0.05$ vs. hypoxic controls

(B) Left Ventricle Cardiac Tissue Lactate



No significant differences were found among groups.

FIGURE 6-10: PLASMA MILRINONE LEVELS

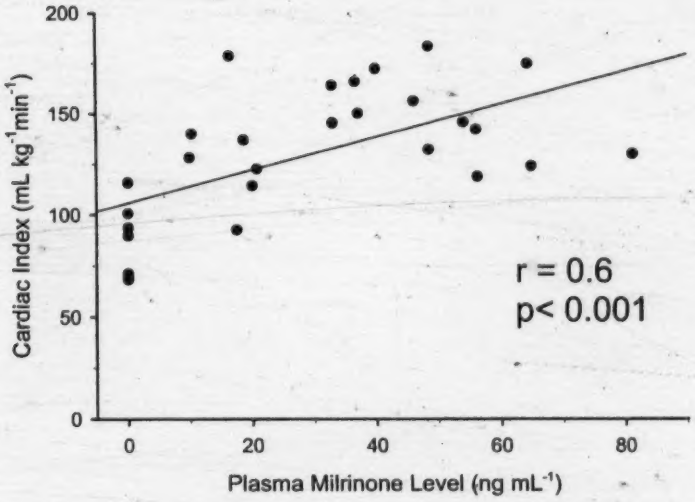


* $p<0.05$ vs. low treatment group

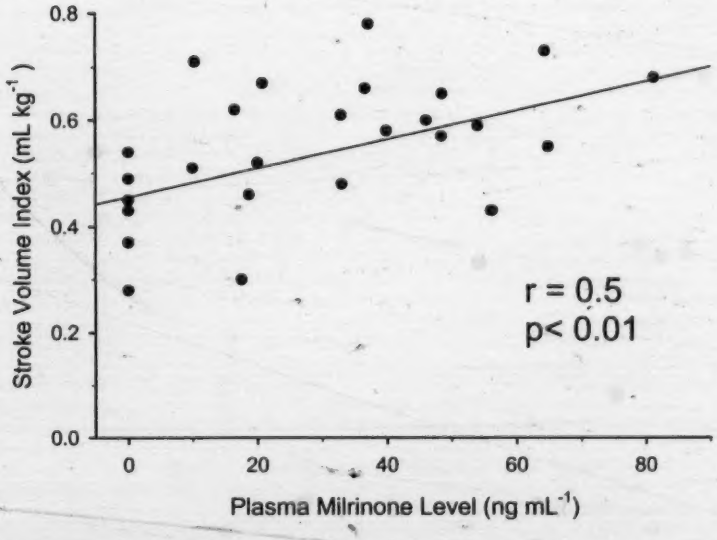
‡ $p<0.05$ vs. mid treatment group

FIGURE 6-11: MILRINONE LEVEL CORRELATES WITH CARDIAC FUNCTION

(A) Cardiac Index vs. Milrinone Level

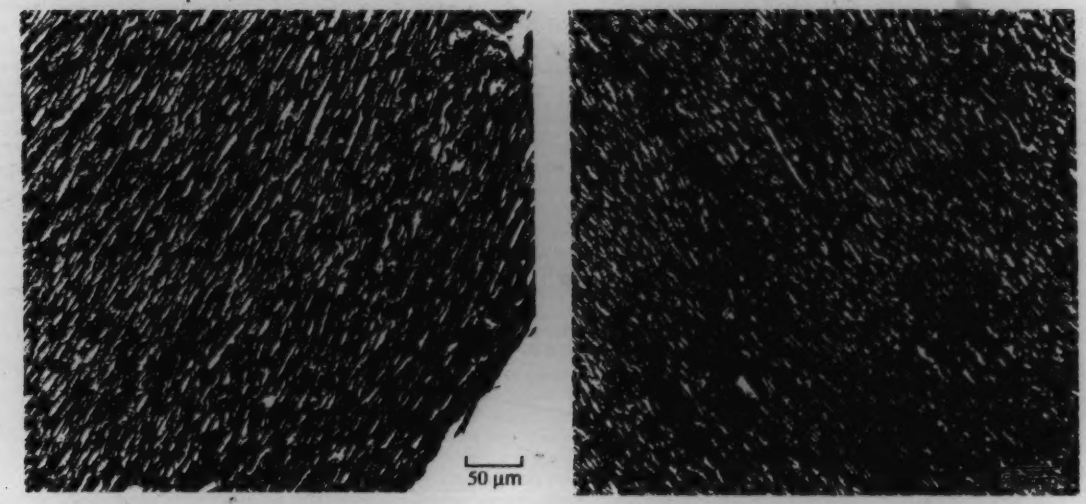


(B) Stroke Volume Index vs. Milrinone Level



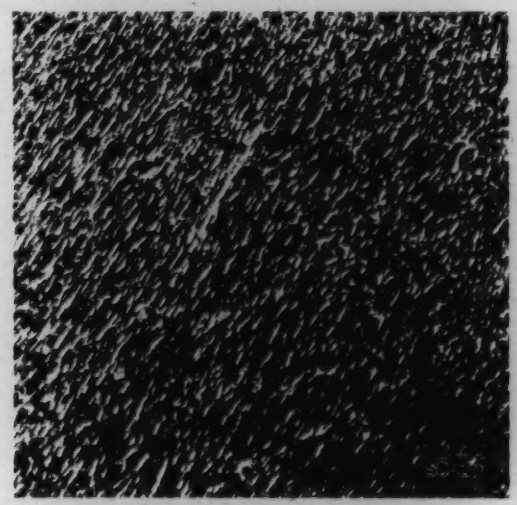
Significant correlations between plasma milrinone levels and (A) cardiac index and (B) stroke volume index after 2h of treatment.

FIGURE 6-12: CARDIAC TISSUE HISTOLOGY



(A) Sham-operated (normoxia) piglet

(B) Hypoxic placebo control piglet



(C) High-dose milrinone treated piglet

All groups of piglet left ventricle myocardium (Hematoxylin and Eosin stain) demonstrated normal myocardium with no significant ischemic changes or necrosis.

DISCUSSION

In this study, we have shown that milrinone (0.25-0.75 μg/kg/min) can increase CI in a dose-related manner in hypoxic-reoxygenated piglets. All doses of milrinone maintained MAP without causing significant hypotension despite reductions in SVRI. Pulmonary vascular resistance index was significantly reduced by all doses of milrinone. All doses of milrinone increased systemic oxygen delivery and consumption without increases in plasma and cardiac tissue lactates. High-dose milrinone improved carotid flow and oxygen delivery with a modest decrease in carotid vascular resistance.

Systemic Hemodynamic Effects of Milrinone

Neonatal asphyxia induces cardiac dysfunction with reduced myocardial contractility and passive dilation leading to cardiac stunning and systemic hypotension.[1,3] This low cardiac output state, similar to that in neonates after cardiac surgery, is evidenced by the continual decrease in cardiac function in the hypoxic placebo control group despite reoxygenation. Milrinone increases myocardial contractility by elevating the intracellular cAMP and calcium concentrations resulting in higher stroke volume and CI. [9,10, 22] Doppler echocardiography in neonates and infants have demonstrated that milrinone has a direct myocardial effect to increase biventricular function and improve low cardiac output.[23] Our findings suggest that, as indicated by increased stroke volume and no significant changes in heart rate, the improvement in CI is inotropic rather chronotropic. However, the chronotropic effect of milrinone may have been masked by the tachycardia resulting from neonatal H-R.[24]

Milrinone's improvement in ventricular function is thought to not be associated with increased myocardial oxygen consumption.[13] During milrinone infusion, Chang *et al*

found no increase in myocardial oxygen consumption.[12] Although we did not measure coronary hemodynamics or perfusion, our study found no significant increase in myocardial tissue lactate in the milrinone treated groups compared to hypoxic controls. We did not find any evidence of histological features of ischemic changes or H-R damage in the left ventricle of these piglets. Given the short duration of the model and gross nature of the biochemical and histological examination, cautious interpretation of these findings is warranted.

We demonstrated a significant correlation between cardiac function and plasma milrinone levels. Two pediatric studies in postoperative cardiac patients, only one of which was predominantly neonatal patients, have determined that milrinone levels of 140-235 ng/mL correlated with significant cardiovascular effects.[12,25] Our study, however, indicated that a cardiovascular effect occurred at much lower plasma milrinone levels in H-R. The average plasma milrinone level in the high-dose group of our study was 58 ng/mL. This may be due to subtle differences in the pathophysiology of low cardiac output syndrome between H-R and post cardiopulmonary bypass patients or due to different milrinone pharmacokinetics between piglets and human neonates.

There is a concern that milrinone may decrease MAP because of vascular smooth muscle relaxation with increased cAMP concentrations.[5,12] Unlike some studies of milrinone in cardiac neonates (n=10) and premature infants at risk for hypotension (n=29), our study did not find an appreciable drop in blood pressure with milrinone load or infusion despite a significant decrease in SVRI.[12,14] Indeed, our findings showed a maintenance of MAP during the milrinone infusion phase similar to studies in pediatric cardiac and PHT patients.[12,26] It is interesting to note, that all piglets received 10 mL/kg

of normal saline 20 min prior to milrinone treatment to ensure circulatory volume. Adequate volume status coupled with the increase in CI most likely helped to maintain the blood pressure despite decreased SVRI.

Pulmonary Hemodynamics Effects of Milrinone

Neonatal asphyxia can cause PHT, which has been previously demonstrated in this model of H-R.[7,27,28] In the pulmonary vasculature, the increase in cAMP concentrations may result in vasodilation that can decrease PVRI. Two recent case series have reported successful milrinone use in the setting of unresponsive PHT, in term and growth restricted preterm infants likely due to the drug's ability to increase ventricular output and decrease pulmonary vascular resistance.[15,16]

This study showed a significant dose-related decrease in PVRI with milrinone and negative correlation between milrinone level and PVRI. No significant change in PAP was observed. Nonetheless, the enhanced cardiac output with milrinone infusion post H-R does not exacerbate PHT as found with vasopressor doses of epinephrine or dopamine.[29,30] Milrinone may have more of an effect on PVRI than SVRI as even the low-dose milrinone significantly affected PVRI.

Systemic Oxygen Metabolism with Milrinone Infusion

In this study, systemic oxygen delivery was significantly improved with all doses of milrinone corresponding to increased CI. The increase in systemic oxygen consumption in milrinone groups with no significant difference in systemic oxygen

extraction is most likely a reflection of improved organ perfusion in contrast to the decreased systemic oxygen consumption of the control animals.

Despite the increase in systemic oxygen delivery and consumption, we did not observe a significant increase in plasma or cardiac tissue lactate levels after 2h of milrinone infusion compared to hypoxic controls. Although we are not certain about the hepatic clearance of lactate, we believe that the increased systemic oxygen consumption did not exceed the increased systemic oxygen delivery and did not seem to be associated with secondary tissue hypoxia or dysfunction in oxygen metabolism that can be found with epinephrine or dobutamine used to treat shock in neonatal H-R.[6,31]

Carotid Hemodynamics and Oxygen Delivery

Cerebral blood flow is often impaired in infants who have sustained hypoxia or neonatal asphyxia.[32] Studies have shown poor cerebral blood flow during resuscitation occurs despite the normalization of blood gas parameters, P_aO_2 and recovery of blood pressures.[33,34] A significant linear correlation ($r = 0.84$) between carotid and cerebral blood flows has been shown in animal models of H-R.[35]

Our study demonstrated that high-dose milrinone (0.75 μg/kg/min) was more effective than lower doses at improving carotid hemodynamics. Milrinone, in very high doses (250 μg/kg), has been shown to increase cerebral blood flow when used to treat hypoxic, but not resuscitated, adult dogs.[36] Despite an increased CI with all doses of milrinone, the addition of a modest decrease in carotid vascular resistance may explain the favorable carotid hemodynamics at high but not lower milrinone doses. This is supported by animal models and preliminary work of superior vena cava flow in neonates

that indicate that peripheral vascular resistance, not blood pressure, may be indicative of regional blood flow post asphyxia.[37] The results of this study would support the findings of Weindling *et al* that cerebral blood flow may be independent of blood pressure and rely more on cardiac output for adequate cerebral tissue function and oxygenation.[38]

Limitations and Clinical Implications

Despite similar cardiovascular changes in newborn piglets and human neonates during H-R, this is an animal study with recognizable limitations. Unlike clinical neonatal asphyxia, the duration and severity of hypoxemia and acidemia was controlled in the presence of artificially regulated P_aCO_2. Our study model approximates the clinical scenario of neonatal asphyxia but is distinct from perinatal asphyxia. Nonetheless, piglets were hypotensive with cardiac stunning and decreased carotid flows after H-R.

Although the piglet's baseline hemodynamics and pathophysiological response to H-R is known to be similar to that of a human neonate, the pharmacodynamics of milrinone is unknown in a piglet.[39,40] Despite this fact, the cardiovascular response of the piglets to milrinone in this study is similar to those reported in neonates with congenital heart disease or PHT.

Although the myocardial contractile response of milrinone appears preserved with ongoing clinical use,[5] this study only investigated short term milrinone infusion. Further long term studies of milrinone infusions looking for beneficial and detrimental effects including comparisons to currently used pressors and inotropes in neonates after H-R injury are warranted.

In conclusion, in a swine model of neonatal H-R, milrinone causes concentration-dependent and effective inotropy for post-asphyxial myocardial dysfunction.

The significant decrease in PVRI may reduce the effects of PHT. Milrinone did not cause significant hypotension. CAFI was improved with higher doses of milrinone and may reflect improved brain perfusion and oxygen delivery.

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CHAPTER 7

The Intestinal Effects of Milrinone

in a Newborn Piglet Model of Hypoxia-Reoxygenation

A version of this chapter has been submitted for publication to Annals of Surgery. Joynt CA, Bigam D, Charrois G, Jewell LD, Cheung PY.

High Performance Liquid Chromatography and sample preparation of plasma for milrinone level determination was performed by Ken Strynadka and John Ussher. Glutathione and intestinal lactate assays were performed by Corinne Tymafichuk.

INTRODUCTION

Poor perfusion and decreased oxygen transport to organs such as the intestinal tract often occur in neonates with asphyxia.[1] Martin-Ancel *et al* evaluated a series of 72 asphyxiated term babies and found 29% had gastrointestinal involvement consisting of abdominal distention, poor feeding, emesis or mild ileus on radiographs.[2] Animal studies have extensively described the redistribution of blood flow during asphyxia.[3-6]

In fetal sheep with asphyxia (25 min), there was persistent mesenteric hypoperfusion associated with increased vascular resistance upon reoxygenation.[7-9] Latent gut perfusion was restored post hypoxia only when mesenteric vascular resistance decreased and not when blood pressure increased.[8] However, our previous study of hypoxia (2h) on the neonatal piglet mesenteric circulation has shown that upon reoxygenation, blood flow increased for 5-15 min to levels well above normoxic baseline with a subsequent decline to baseline values with decreased vascular resistance.[10]

Current treatments for post asphyxial shock include catecholamines, such as dopamine and epinephrine, which at high doses can further increase peripheral vascular resistance. Particularly, the catecholamine treatment may stress tissue oxygenation without significantly increasing mesenteric blood flow or oxygen delivery.[11,12]

Milrinone is a specific phosphodiesterase (PDE) III inhibitor that increases cardiac output and also produces vasodilation secondary to increased intracellular cyclic adenosine monophosphate (cAMP) concentration. The literature on the effect of milrinone in the gastrointestinal system is sparse. Studies of the isolated stomach wall of the guinea pig incubated with milrinone have shown no adverse effect on gastric ion transport.[13] Further studies on adults after cardiopulmonary bypass studies confirmed

that low-dose IV milrinone (0.25 μg/kg/min) did not negatively impact gastric pH, an indirect measure of gastric mucosa perfusion.[14] The use in neonatal patients is predominantly for the improvement of a low cardiac output state.[15,16] However, there is no literature on how milrinone affects neonatal intestinal hemodynamics.

Using a swine model of neonatal hypoxia-reoxygenation (H-R), the primary objective was to examine the dose-dependent effects of milrinone on mesenteric hemodynamics. To investigate if milrinone-induced hemodynamic changes have any effect on the intestine, we also examined the intestine regarding the effects on oxygen transport, oxidative stress and histologic features of reoxygenation injury. We hypothesized that milrinone would dose-dependently increase intestinal blood flow after hypoxia-reoxygenation with a decrease in the vascular resistance in newborn piglets.

METHODS

Mixed breed piglets 1-3 days of age weighing 1.5-2.3 kg were obtained from a local farm on the day of experimentation. The experimental protocol was approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee.

Anesthesia

The piglets were initially anesthetized with 5% halothane and maintained at 2-3%. Inhalational anesthesia was discontinued once mechanical ventilation via a tracheostomy was initiated. Anesthesia, analgesia, and paralysis were maintained with intravenous midazolam (0.1-0.2 mg/kg/h), fentanyl (5-15 μg/kg/h) and pancuronium (0.05-0.1 mg/kg/h) respectively, with up to two boluses of acepromazine (0.25 mg/kg). Inspired oxygen concentration (FiO_2) was measured by an Ohmeda 5100 oxygen monitor (Ohmeda Medical, Laurel, MD) and maintained at 0.21-0.24 to keep oxygen saturations between 90-100%. Oxygen saturation was measured by continuous pulse oximetry (Nellcor, Hayward, CA). Blood pressure and heart rate were monitored with a Hewlett Packard 78833B monitor (Hewlett Packard Co., Palo Alto, CA). An intravenous 10% dextrose infusion at 10 mL/kg/h maintained glucose levels and hydration. An arterial line was maintained with an infusion of 0.9% normal saline at 4 mL/h. Body temperature was kept at 38.5-39.5°C using an overhead warmer and heating pad.

Surgical Procedure

A 5 F Argyle® double lumen catheter (Sherwood Medical Co., St Louis, MO) was inserted to the level of the right atrium via the femoral vein to measure to deliver

fluids and medications. A 5 F Argyle® single lumen catheter was inserted into the right femoral artery, advanced into the infrarenal aorta and connected to a pressure transducer to continuously measure mean arterial pressure (MAP). Endotracheal intubation via a tracheostomy allowed pressure control assisted mechanical ventilation by an infant ventilator (model IV-100, Sechrist Industries Inc., Anaheim, CA) at a rate of 18-20 breaths/min and pressure of 19/4 cm H_2O.

The retroperitoneum was opened through a left flank incision, and the superior mesenteric artery (SMA) was isolated with minimal dissection and encircled by a 3-mm Transonic® flow probe (3SB, Transonic Systems Inc, Ithica, NY) to measure blood flow. Through a left anterior thoracotomy, the main pulmonary artery was exposed. The ductus arteriosus was ligated. A 6-mm Transonic® flow probe (6SB) was placed around the base of the main pulmonary artery to monitor the blood flow as a surrogate for cardiac output.

Incisions were covered and kept moist to minimize evaporative losses. Transonic® flow probes were connected to a T206 2-channel small animal flow meter. Transonic® flow probe and pressure transducer outputs were recorded and digitized by a DT2801-A analogue to digital converter board (Data Translation, Ontario, Canada) in a Dell 425E computer equipped with custom Asyst programming software.

Hypoxia-Reoxygenation and Treatment Procedure

Piglets were block randomized to 4 groups (n=7/group) which underwent H-R. A sham operated group of piglets (n=4) underwent instrumentation and stabilization without H-R or medication delivery.

After instrumentation, the animals underwent a period of stabilization for 40 min. Stability was defined as a hemodynamic measurement change ±<10% over 20 min, pH 7.35-7.45, P_aO_2 of 60-100 mmHg, and P_aCO_2 of 35-45 mmHg. Normocapnic alveolar hypoxia was initiated with the inhalation of a mixture of oxygen and nitrogen gas to obtain a FiO_2 of 0.10-0.15 and P_aO_2 of 20-40 mmHg for 2h. Previous studies have determined that this degree of hypoxemia in this piglet model will produce a clinical asphyxia with severe metabolic acidosis, systemic hypotension, and a decrease in cardiac index (CI) to 40% of baseline.[17,18] Piglets were then reoxygenated with 100% oxygen for 1h and a further 3h of 21% oxygen. All piglets received a 10 mL/kg normal saline bolus 30 min prior to medication delivery. At 2h of reoxygenation, piglets received a 2h blinded treatment with an intravenous infusion of placebo (normal saline) or high-dose milrinone (75 μg/kg loading, 0.75 μg/kg/min infusion) , mid-dose milrinone (50 μg/kg, 0.75 μg/kg/min), or low-dose milrinone (25 μg/kg, 0.25 μg/kg/min) (Appendix 2).

Medication Preparation and Delivery

To maintain blinding, all doses of milrinone (Milrinone Lactate, Pharmaceutical Partners of Canada Inc., Richmond Hill, ON) and normal saline were reconstituted in a standard volume immediately prior to administration. The medication was infused at 6.7 mL/kg/h for 15 min (medication loading bolus) followed by 1 mL/kg/h for the remainder of 2h. Medication infusions were mixed by a laboratory technician uninvolved in the experiment. All medications were clear, odorless and identified by number only.

Hemodynamic and Oxygenation Measurements

Systemic (heart rate, pulmonary artery blood flow, MAP) and intestinal hemodynamic parameters (SMA blood flow), blood gases and co-oximetry were recorded at post surgical stabilization (0 min), every 30 min during hypoxia, at 0, 10, 30, 60, and 120 min of reoxygenation, and every 30 min following medication or placebo delivery (Appendix 3). Variables were calculated as a mean over 2 min at these specified time points. Hemodynamic variables were calculated as shown in Appendix 4.

At specified time points, simultaneous arterial and venous blood samples were taken for blood gases, hemoglobin measurement, and co-oximetry by ABL500 (Radiometer, Copenhagen, Denmark) and OSM3 Hemoximeter (Radiometer) respectively. The mesenteric oxygen delivery ($SMADO_2$) and estimated SMA vascular resistance index (SMAVRI) calculations are listed in Appendix 4.

Arterial blood samples (1 mL) were taken at predetermined times, centrifuged at 15,000 rpm for 10 min, and the supernatant collected and frozen at -80°C for plasma lactate determination. Less than 5% of the piglet blood volume was collected as bloodwork.

Small Intestine Tissue Collection

At the end of the study, piglets were euthanized with 100 mg/kg pentobarbital IV. Samples of distal ileum were snap frozen in liquid nitrogen and stored at -80°C while another sample was fixed in 10% formalin for subsequent biochemical and histologic analysis, respectively.

Lactate as a Marker of Anaerobic Metabolism

Plasma levels of lactate were determined using a NAD enzyme-coupled colormetric assay. Plasma samples (15 µL) were diluted in ddH_2O (110 µL). Colormetric microplate assay was performed with the addition of glycylglycine buffer, pH 10, NAD, ddH_2O, glutamate-pyruvate transaminase, and lactate dehydrogenase. The absorbance was read at 340 nm with a microplate spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, CA). The lactate concentration was calculated from a standard equation (Appendix 5).

For small intestine tissue lactate determination, frozen intestine tissue (50 mg) was crushed at -80°C and then homogenized in 6% perchloric acid/0.5 mM EGTA (500 µL) on ice. After centrifuging at 11,000 rpm for 2 min at 4°C, the supernatant was weighed and 5 M potassium carbonate was added slowly in a ratio of 1µL:10 µL of supernatant. Precipitation on ice for 30 min was followed by centrifuging at 11,000 rpm for 2 min (Appendix 6). The resulting supernatant was then used in substitute of plasma in the lactate assay described above.

Histopathology

Small intestine samples preserved in formalin were prepared for histological assessment using hematoxylin and eosin staining. Two independent pathologists (Jewell LD, Charrois G) who were blinded to treatment group evaluated histologic damage of the specimens and assigned a grade based on Park's classification of ischemic intestinal injury.[19]

Plasma Milrinone Levels

Plasma samples previously stored at -80°C were used in a high performance liquid chromatographic validated method for the determination of milrinone in plasma.[20] Samples (100 µL) were mixed with internal standard (amrinone 10 µg/mL), 0.15 mL of water and 0.35 mL of acetonitrile and centrifuged at 3,800g x 3 min. The supernatant was collected and 3 mL of 95:5 diethyl ether: methanol was added followed by centrifugation at 3,800g x 3 min again. The organic solvent layer was transferred and dried in vacuo, then reconstituted with 150 µL of the mobile phase (90:7:30 [25 mM KH_2PO_4: 3 mM sulfuric acid: 3.6 mM triethylamine]: methanol: acetonitrile). Samples were chromatographed on a C18 column at 21°C. Detection of milrinone and the internal standard was achieved by UV detection at 326 nm. Signals from the detector were collected and recorded. Standard of milrinone (0-2000 ng/mL) were used as standards. The mean r^2 for standard curves in human plasma is $0.999 \pm 1.9 \times 10^{-5}$. The limit of quantitation was 10 ng/mL.

Intestinal Tissue Glutathione Content

Intestinal levels of total and oxidized glutathione (GSSG) were measured using a glutathione assay kit (catalog no. 703002, Cayman Chemical, Ann Arbor, MI). Samples of ileal tissue previously frozen at -80°C were homogenized with 1 mL/100 mg of buffer containing 0.2 M 2-N-morpholino ethanesulphonic acid, 50 mM phosphate, and 1 mM EDTA, pH 6.0. Homogenates were centrifuged at 10,000g for 15 min at 4°C, and the supernatant was collected and deproteinated with 10% metaphosphoric acid and 4 M triethanolamine, to avoid interference from sulfhydryl groups on proteins in the sample.

A colorimetric microplate assay was performed by adding glutathione reductase, $NADP^+$, and 5,5'-dithiobis-2-nitrobenzoic acid to the sample. The absorbance was measured after 25 min at 405 nm with a microplate reader (Spectra Max 190, Molecular Devices, Sunny Vale, CA), and the total glutathione concentration was calculated from a standard curve. To measure GSSG, deproteinated samples were incubated at room temperature for 1h with 1 M 2-vinylpyridine to completely derivatize the reduced glutathione in the sample, and the colorimetric assay was carried out as above. Glutathione redox status was obtained by calculating the ratio of GSSG:total glutathione.

Statistics

Results are expressed as mean ± standard error of the mean. Hemodynamic variables were analyzed by 2-way ANOVA followed by 1- way Repeated Measures (RM) ANOVA for differences within groups over time and 1-way ANOVA for differences between groups at a given time point. Biochemical markers were analyzed by 1-way ANOVA. If the normality test failed, ANOVA on ranks (Kruskal-Wallis) was performed. We used the Tukey or Dunn's method where appropriate for pairwise comparisons in post hoc testing. Pearson Moment correlation was used to determine the relationship between hemodynamic and biochemical variables. Analysis was performed using Sigma Stat (Version 2.0, Jandel Scientific, San Rafael, CA). Significance was defined as $p<0.05$.

RESULTS

There were no significant differences among the groups with respect to weight and age with an average age of 2.0±0.2 days old and weight of 1.86±0.04 kg. The mortality was 11% (4/36 piglets) secondary to surgical complications (2) and severe irreversible hypoxia (2).

Hypoxia

At 2h of normocapnic alveolar hypoxia there was a metabolic acidosis with a pH 7.04±0.04, P_aO_2 34±1 mmHg, and arterial oxygen saturations of 34-40% (Table 7-1). The cardiac index (CI) decreased to 71±4 mL/kg/min (41±1% of the normoxic baseline) with accompanying hypotension (MAP of 28±2 mmHg; 41±2%) (Table 7-2). Superior mesenteric artery flow index (SMAFI) was reduced to 14±1 mL/kg/min (41±4%) (Figure 7-1). Superior mesenteric artery oxygen delivery ($SMADO_2$) decreased to 13±1% (Figure 7-2). At the end of hypoxia, the superior mesenteric artery vascular resistance index (SMAVRI) was not different from normoxic baseline (98±8%). There were no significant differences in hemodynamic parameters among hypoxic groups.

Reoxygenation

Upon reoxygenation with 100% oxygen, hemodynamics immediately recovered to baseline as measured at 10 and 30 min. This recovery was not sustained, as the hemodynamic parameters gradually deteriorated over the next 90 min of reoxygenation. Arterial oxygen saturation (88-92%), P_aO_2 (61±1 mmHg), and pH (7.38±0.01) normalized but MAP was decreased to 67±3%, and CI to 75±3% of the respective

normoxic baseline ($p<0.05$ vs. normoxic baseline) (Table 7-2). After 2h of reoxygenation, SMAFI decreased to 88±5%, $SMADO_2$ 84±5%, and SMAVRI 76±6% of normoxic baseline (Figures 7-1, 7-2). No significant differences in hemodynamic parameters were found among groups prior to medication delivery.

After Milrinone or Placebo Infusion

After 2h of medication or placebo infusion, the arterial blood gas was within normal parameters (oxygen saturations 90-92%, P_aO_2 65±2 mmHg, and pH 7.40±0.01). Milrinone increased CI in a dose-related manner compared to that of hypoxic placebo control. There was no significant difference in heart rate and MAP among milrinone and placebo control groups after 2h of treatment (Table 7-2).

Superior Mesenteric Circulation

Following the infusion, SMAFI increased in the milrinone groups whereas it gradually declined in the hypoxic placebo control groups ($p<0.05$, high-dose milrinone group; $p<0.1$, $\beta=0.4$,mid and low-dose milrinone groups) (Figure 7-1). Superior mesenteric artery oxygen delivery was significantly increased with the high-dose milrinone infusion, with a modest effect in mid and low-dose milrinone groups (vs. hypoxic controls $p<0.1$, $\beta=0.5$).

While SMAFI and $SMADO_2$ values returned to their respective normoxic baseline values after 2 h of milrinone treatment, there were significant drops in SMAFI and $SMADO_2$ in the hypoxic placebo control group ($p<0.05$ vs. normoxic baseline) (Figure 7-2).

In the high-dose milrinone group, there was a trend towards decreasing SMAVRI compared to the control group ($p=0.06$, $\beta=0.6$, ANOVA; $p < 0.05$, t-test) (Figure 7-3). Compared to normoxic baseline, milrinone treatment at all doses produced significantly lower SMARVI ($p<0.05$). This decrease was not found in the hypoxic control group.

Plasma Milrinone Levels

Plasma milrinone levels showed a significant dose-response between the groups (low-dose:16±2, mid-dose:43±4, high-dose:58±5 ng/mL; $p<0.05$ between the groups). Plasma milrinone levels correlated with SMAFI ($r=0.5$, $p<0.05$) and negatively with SMAVRI ($r=-0.6$, $p<0.001$) (Figure 7-4). A negative correlation was found between the plasma milrinone levels and GSSG in the intestinal tissue ($r=-0.5$, $p<0.01$).

Intestinal Total and Oxidized Glutathione Levels

No significant differences in total glutathione, GSSG, or GSSG:total glutathione ratio were found among treatment groups. A significant correlation was found between SMAVRI and GSSG ($r = 0.6$, $p<0.005$), but not with the GSSG:total glutathione ratio (Figure 7-5).

Intestinal Tissue Lactate Levels

The tissue lactate levels of the small intestine did not show a significant difference in lactate levels among groups (Figure 7-6).

Histology

There were no significant differences in histology samples found between groups. Only one sample in the mid-dose milrinone group demonstrated histological changes with a small patch of denuded villi that was surrounded by normal mucosa (Figure 7-7). One piglet in the low-dose milrinone group had visible intestinal pneumatosis at the time of autopsy.

TABLE 7-1: ARTERIAL BLOOD GASES

Variable	Normoxic baseline	End of hypoxia	Pretreatment baseline	1 hour of treatment	2 hours of treatment
pH					
Control	7.40±0.01	7.04±0.02#	7.38±0.03	7.39±0.02	7.39±0.03
Low-Dose	7.40±0.02	7.01±0.03#	7.37±0.02	7.38±0.01	7.40±0.02
Mid-Dose	7.40±0.01	7.00±0.04#	7.38±0.02	7.39±0.02	7.37±0.02
High-Dose	7.43±0.01	7.10±0.03#	7.41±0.02	7.42±0.01	7.42±0.02
P_aO_2 (mm Hg)					
Control	60±3	35±3#	62±4	55±4	63±6
Low-Dose	65±3	39±3#	61±2	69±3*	73±5
Mid-Dose	72±10	35±2#	62±2	62±3	64±2
High-Dose	64±2	31±2#	60±2	65±2	58±1
P_aCO_2 (mm Hg)					
Control	42±1	42±2#	37±1	40±1	40±1
Low-Dose	41±1	37±2#	39±1	39±1	39±1
Mid-Dose	40±1	42±3#	38±1	39±1	40±2
High-Dose	39±1	43±2#	39±2	39±2	39±1
HCO_3^- (mmol/L)					
Control	25±1	11±1#	22±1	23±1	25±2
Low-Dose	25±1	9±1#	22±1	23±1	23±1
Mid-Dose	24±1	10±1#	22±1	23±1	23±1
High-Dose	26±1	13±1#	24±1	25±1	25±1

* $p<0.05$ vs. control (ANOVA)

# $p<0.05$ vs. normoxic baseline (RM ANOVA)

TABLE 7-2: SYSTEMIC HEMODYNAMIC VARIABLES

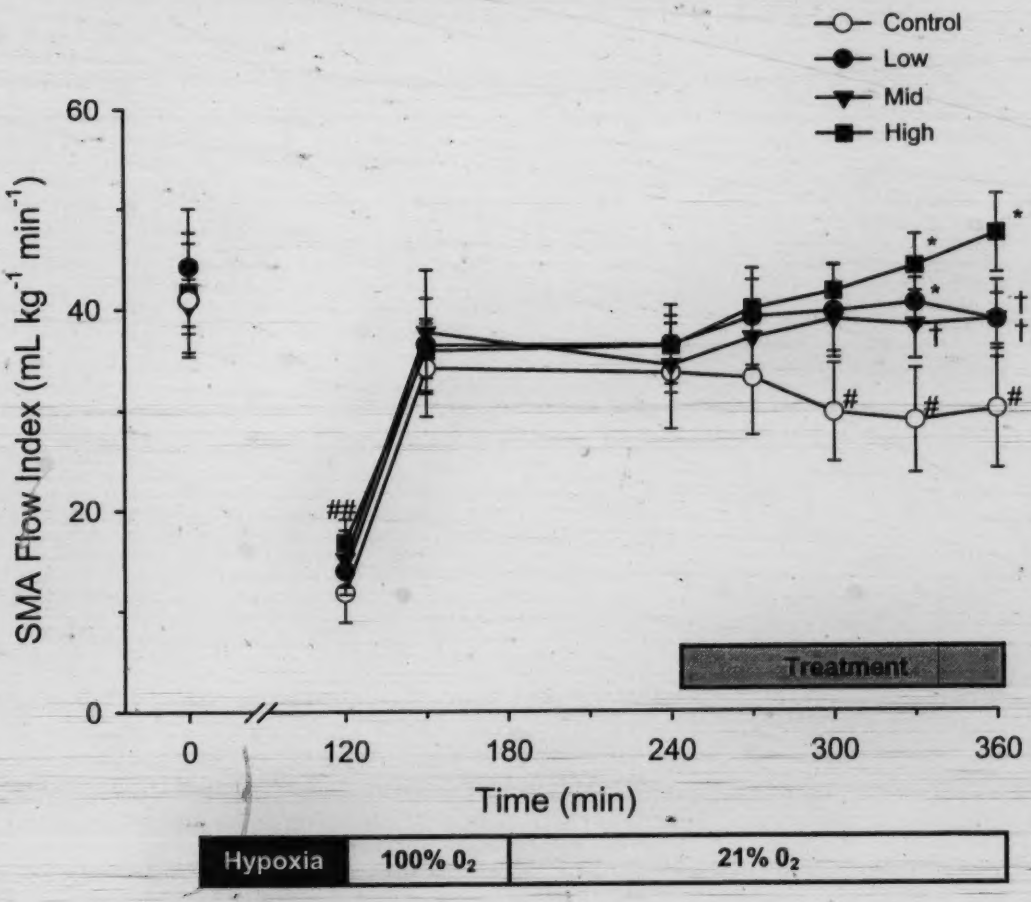
Variable	Normoxic Baseline	End of Hypoxia	Pretreatment Baseline	1 hour of treatment	2 hours of treatment
Heart Rate (beats/min)					
Control	187±6	203±14	215±15	229±11#	219±11#
Low-Dose	208±17	222±11	222±16	247±20#	250±19#
Mid-Dose	204±14	191±15	243±9	253±7#	260±17#
High-Dose	183±10	205±19	203±16	229±12#	253±14#
Mean Arterial Pressure (mm Hg)					
Control	68±4	29±3#	39±2#	39±3#	38±3#
Low-Dose	73±5	30±1#	47±3#	49±2#	46±2#
Mid-Dose	66±4	26±1#	48±3#	45±3#	47±2#
High-Dose	73±5	28±2#	42±2#	43±2#	44±2#
Cardiac Index (mL/kg/min)					
Control	160±16	62±8#	110±10#	100±10#	87±7#
Low-Dose	171±11	70±5#	127±3#	134±7	130±10*
Mid-Dose	191±20	78±9#	144±6#	147±9	149±5*
High-Dose	172±5	73±3#	126±10#	141±8	151±10*†

* $p<0.05$ vs. control (ANOVA)

† $p<0.1$ vs. low-dose milrinone infusion group (ANOVA)

# $p<0.05$ vs. normoxic baseline (RM ANOVA)

FIGURE 7-1: SUPERIOR MESENTERIC ARTERY (SMA) FLOW INDEX

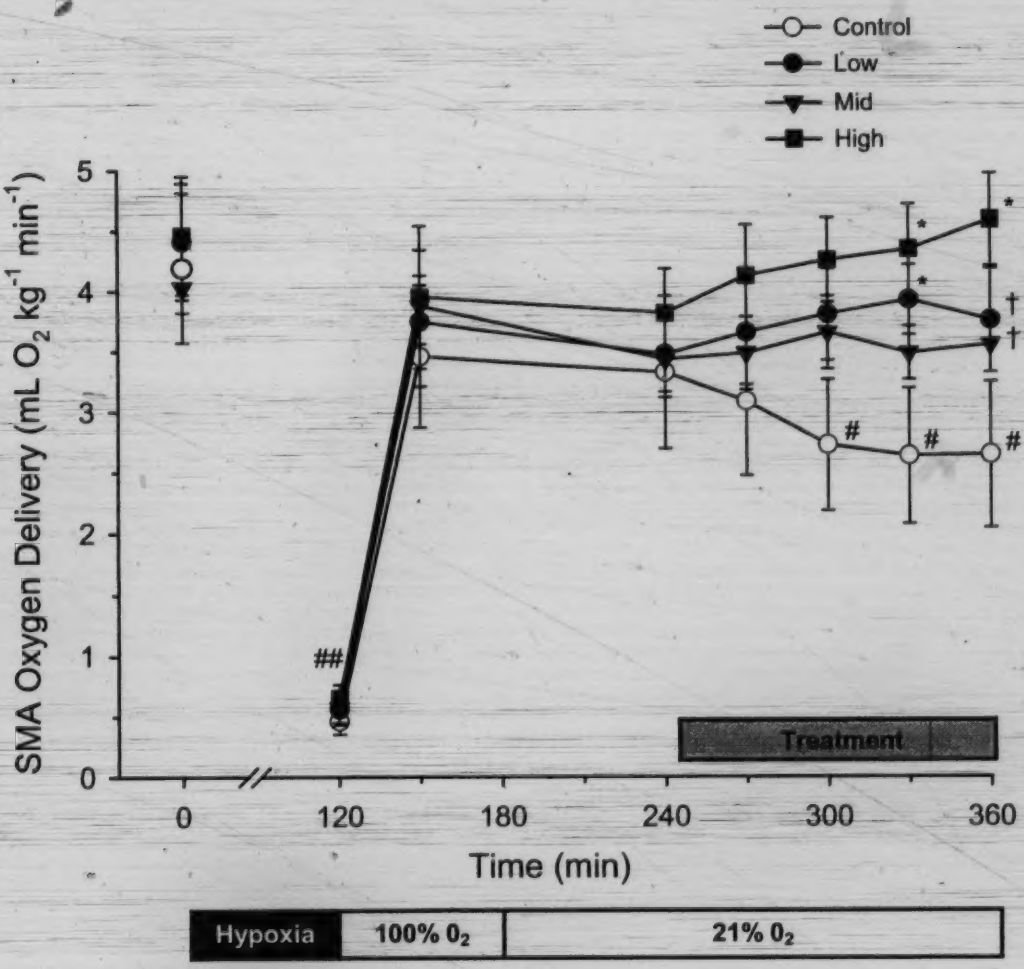


* $p<0.05$ and † $p<0.1$ vs. controls (ANOVA)

\# $p<0.05$ vs. normoxic baseline at 0 min (RM ANOVA)

\## $p<0.05$ all groups vs. normoxic baseline at 0 min (RM ANOVA)

FIGURE 7-2: SUPERIOR MESENTERIC ARTERY (SMA) OXYGEN DELIVERY

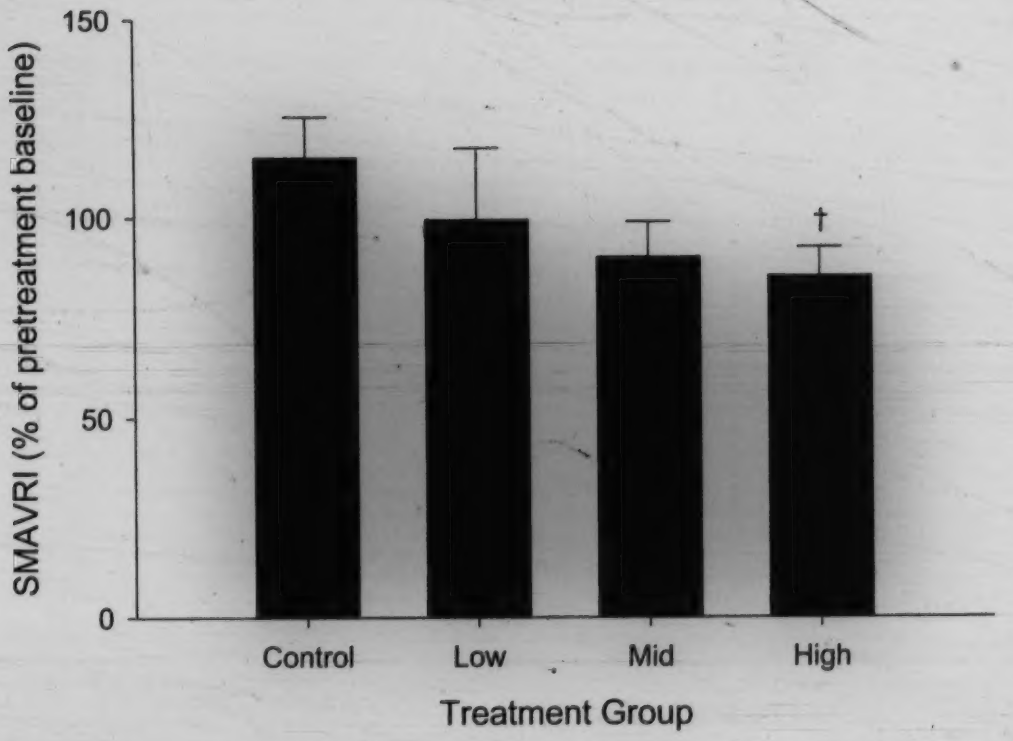


* $p<0.05$ and † $p<0.1$ vs. control group (ANOVA)

# $p<0.05$ vs. normoxic baseline (RM ANOVA)

##$p<0.05$ all groups vs. normoxic baseline (RM ANOVA)

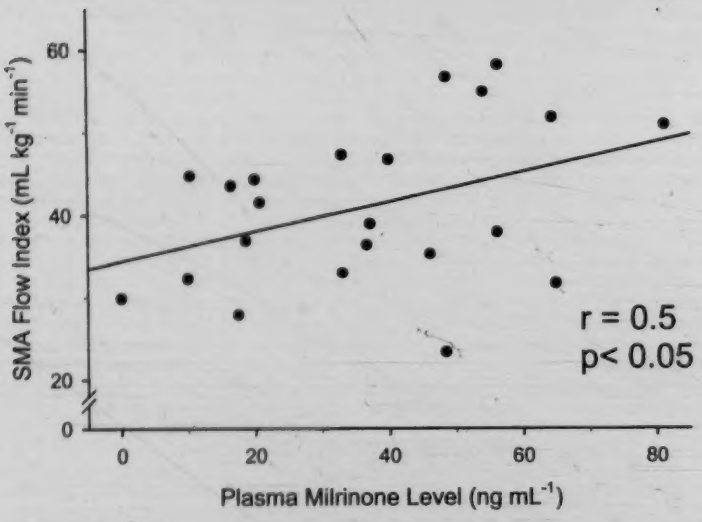
FIGURE 7-3: SUPERIOR MESENTERIC ARTERY VASCULAR RESISTANCE INDEX (SMARVI)



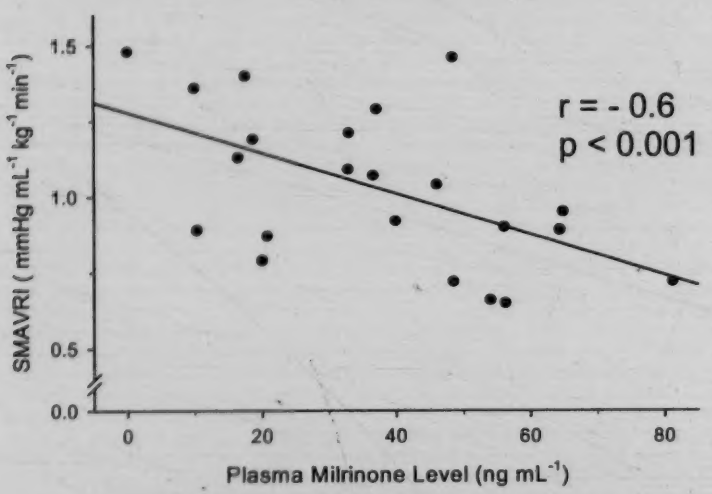
† p= 0.06 vs. control group (β=0.6, ANOVA; p<0.05, t-test)

FIGURE 7-4: MILRINONE LEVELS CORRELATE WITH INTESTINAL HEMODYNAMICS

(A) Superior Mesenteric Artery (SMA) Flow Index



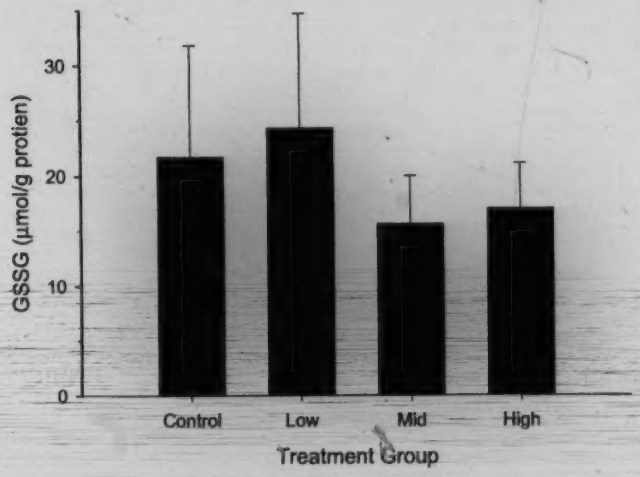
(B) Superior Mesenteric Artery Vascular Resistance Index (SMAVRI)



Pearson correlation shows a significant correlation between plasma milrinone level and (A) SMA flow index (B) SMAVRI after 2h of treatment.

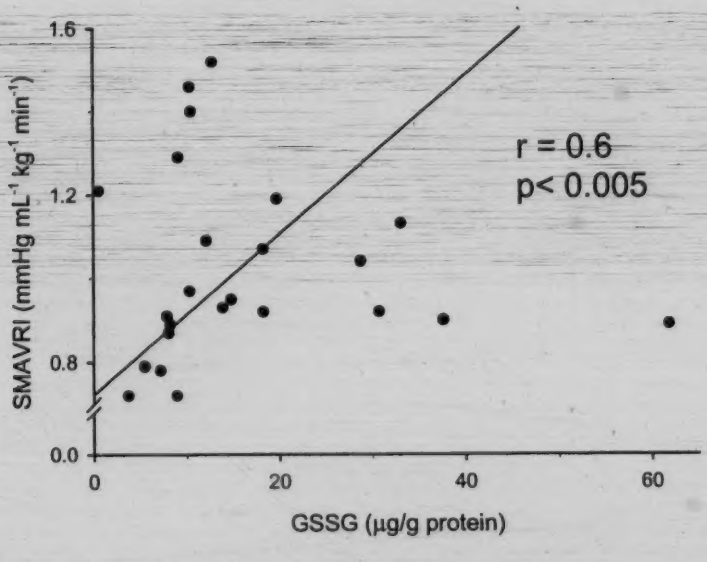
FIGURE 7-5: INTESTINAL GLUTATHIONE

(A) Oxidized Glutathione (GSSG) and Treatment Group



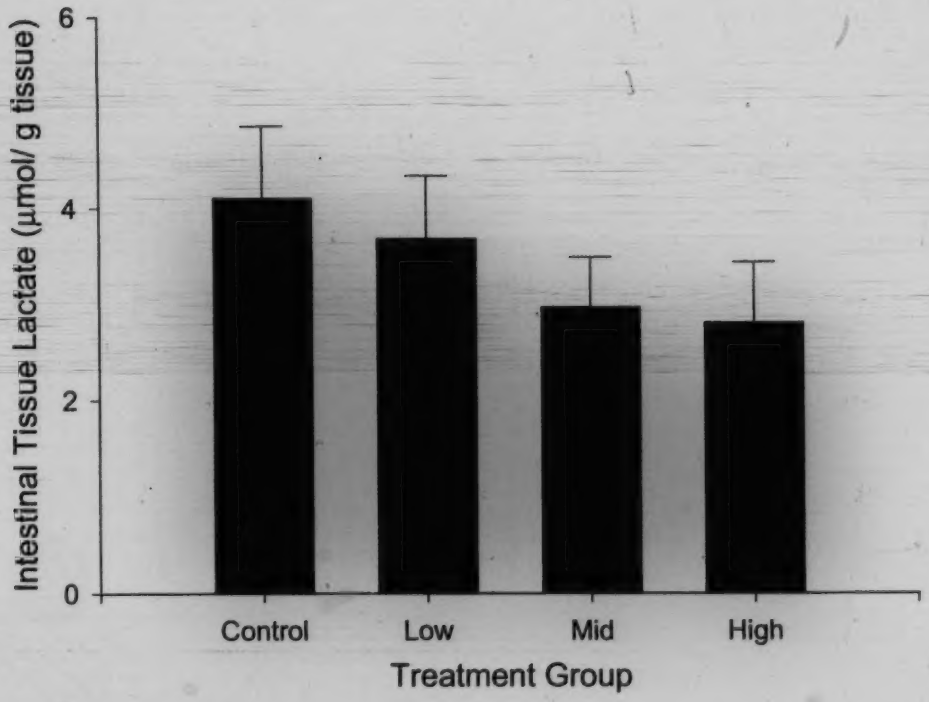
There is no significant difference in GSSG among groups.

(B) Oxidized Glutathione (GSSG) and Superior Mesenteric Artery Vascular Resistance Index (SMARVI)



Pearson correlation shows a significant correlation between GSSG and SMAVRI.

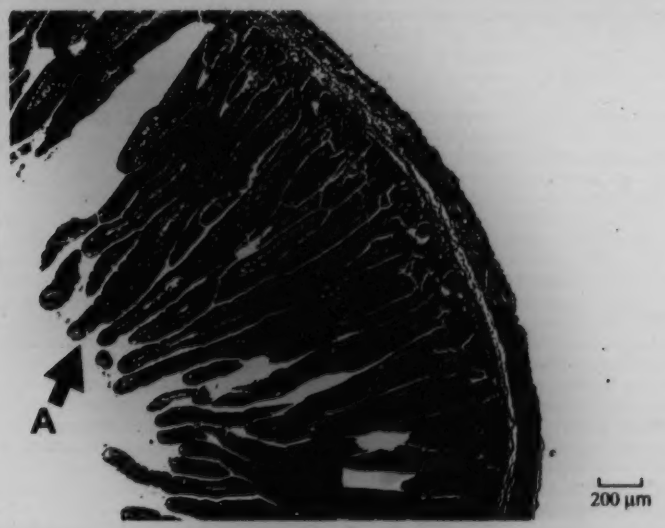
FIGURE 7-6 : INTESTINAL TISSUE LACTATE LEVELS



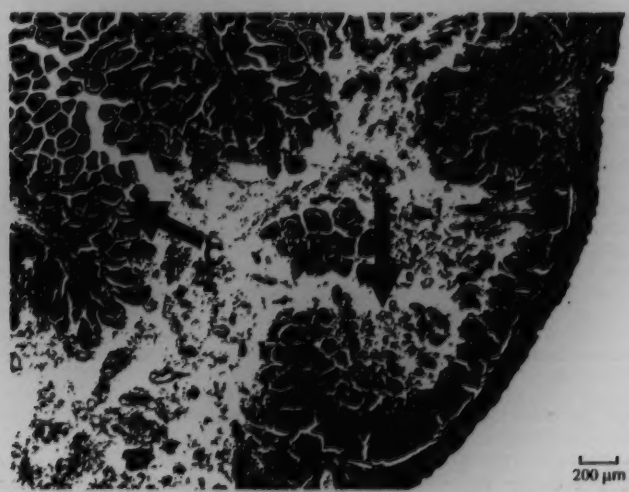
No significant differences were found among the groups.

FIGURE 7-7 : INTESTINAL TISSUE HISTOLOGY

(A) Normal Intestinal Villi of a Piglet in the High-Dose Milrinone Group



(B) Denuded Intestinal Villi of a Piglet in the Mid-Dose Milrinone Group



Histology of piglet intestine (high-dose milrinone group) showing normal villous structure (arrow A). One piglet (mid-dose milrinone group) had an area of denuded villi (arrow B) surrounded by normal villi (arrow C). No other intestinal injury was found on pathology. There were no significant differences in pathology among treatment groups.

DISCUSSION

During asphyxia, the neonate preserves vital organ perfusion, mainly to the heart and brain, at the expense of other organs such as the gut.[1] Intestinal injury in term neonates occurs with an incidence of 0.16-0.71 per 1000 live births,[21] and up to 29% in those with perinatal asphyxia.[2] Studies in asphyxiated newborn piglets have shown decreased blood flow and oxygen delivery, with or without an increased vascular resistance, to the gastrointestinal system during severe hypoxia.[5,6,10,22] These hemodynamic changes are associated with mucosal necrosis, interstitial hemorrhage, and pneumotosis intestinalis. Improving the blood flow and reducing the vascular resistance to the gastrointestinal tract after severe hypoxia has been associated with decreased histologic injury.[4] In our study, we demonstrated a concentration-dependent increase of SMAFI with milrinone treatment. In addition, milrinone at 0.75 μg/kg/min decreased the SMA vascular resistance compared to the gradual increase found with hypoxic placebo control treatment. This may be beneficial to the recovery of the intestine from the H-R insult.

The effects of H-R on SMAFI and SMAVRI are not clear in the literature. Videomicroscopy in acutely hypoxic piglets (30 min) has shown that decreased mesenteric flow during hypoxia coincided with a significant vasoconstrictive response with a return to normal vessel diameter and blood flow upon reoxygenation.[23] However, other hypoxic animal models demonstrate persistent intestinal hypoperfusion associated with increased vascular resistance despite reoxygenation. Restoration of gut perfusion after hypoxia occurred only when SMA vascular resistance decreased and not when blood pressure increased.[7,8]

While high-dose milrinone infusion caused a significant increase in SMAFI over the control, our findings also support a dose-related response as evidenced by the modest changes at lower doses (0.25-0.50 μg/kg/min) and the positive correlation between the flow and plasma milrinone levels. The changes in mesenteric blood flow are related to the changes in regional vascular resistance, and at least in part, to the concomitant changes in the CI. A dose-related effect of milrinone on the CI was also observed in this study. The relationship between blood pressure, CI, and intestinal perfusion during H-R is still not well defined.[7,8,23,24] In our study, milrinone's ability to support the intestinal perfusion coincided with an increased CI during a time period where there was no signicant change in MAP. Thus, our data would support the findings of others,[7,8] that hemodynamic factors other than blood pressure contribute to restoring intestinal perfusion post H-R.

The small sample size of this study precludes us to detect significant changes in SMARVI (β=0.6). High-dose milrinone infusion caused a slight decrease in SMAVRI. A significant negative correlation was observed between SMAVRI and plasma milrinone levels, supporting a concentration-response effect of milrinone on the mesenteric vasculature. Milrinone's ability to increase cAMP and cGMP via PDE-III inhibition may augment mesenteric vasodilation provided by nitric oxide during H-R.

This study demonstrated that increased SMARVI was associated with increased GSSG. Increased GSSG may indicate the presence of oxygen free radicals generated by H-R in neonatal intestine. Reactive oxygen species can alter the balance between nitric oxide and endothelin -1 in the intestine to favor vasoconstriction, leading to further intestinal ischemia and reperfusion injury.[25] Increased milrinone levels correlate with

decreased GSSG levels. We are not certain as to the cause of this relationship between milrinone treatment and oxidative stress. Interestingly, Hayashide *et al* have shown that milrinone inhibited pro-inflammatory cytokine formation (tumour necrosis factor alpha), thus dampening H-R injury.[26]

Treatment of the clinical manifestations of shock and mesenteric hypo-perfusion after asphyxia includes medications such as dopamine, dobutamine and epinephrine. However, these catecholamines or sympathomimetics in clinically relevant doses do not increase SMAFI or decrease SMAVRI, although some may increase CI with or without increased MAP.[17,27-31] In a prospective non blinded study, 20 preterm infants with prolonged hypotension were given either 10 μg/kg/min of dopamine or dobutamine. Both medications significantly increased the blood pressure with increased SMA blood flow velocity and decreased SMAVRI, as measured by Doppler ultrasonography.[24] This study involved preterm hypotensive infants who often have different etiologies for intestinal injury compared to asphyxiated term infants. Further studies to compare the intestinal effects of milrinone to currently used inotropes and pressors, such as dobutamine and epinephrine, are required.

Samples of intestine taken at the end of treatment did not show an increase in gut tissue lactate compared to hypoxic controls indicating a lack of hypoxic stress. Further, the markers for oxidative stress were not different among the groups. Lack of intestinal damage on histology may indicate a premature analysis and should be interpreted with caution. Histopathology changes of intestine after asphyxia may take up to 24h to become apparent. Intestinal necrosis and injury in term infants usually develops within days of birth, implicating perinatal events such as primary asphyxia, cardiac dysfunction, low

cardiac output, and hypoxic ischemic injury as plausible pathogenic factors in these infants.[21,32-35]

Limitations of this study design include the fact that piglets, in comparison to human neonates, may metabolize milrinone differently with possible alterations in receptors and second messenger cell signaling. As this was an acute instrumentation study, we do not know if prolonged milrinone infusions would be detrimental or beneficial to gut perfusion or oxygen use.

In conclusion, milrinone concentration-dependently increased SMA flow and oxygen delivery with significantly decreased SMAVRI at higher doses. This potentially advantageous effects of milrinone warrant further study.

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CHAPTER 8

Renal Hemodynamic Effects of Milrinone in a Newborn Piglet Model of Asphyxia-Reoxygenation

INTRODUCTION

Multiorgan dysfunction in neonatal asphyxia is thought to be secondary to the redistribution of blood flow away from non-vital organs that occurs during asphyxia, resulting in hypoxia-ischemia related dysfunction in one or more of these organs. Among these organs, the kidneys are commonly affected by hypoxia. Human observational studies have demonstrated a 40-70% rate of renal involvement in asphyxiated term neonates.[1-3] Neonatal animal models demonstrate a rapid decrease in renal blood flow with increased vascular resistance during hypoxia[4,5]. Decrease in renal blood supply results in tissue hypoxia, leading to necrosis of renal tubular cells, or acute renal failure.[6]

Despite decreased renal perfusion, there is no conclusive evidence that current medications for shock post asphyxia provide a benefit to the renal vasculature. Animal and clinical studies have demonstrated little effect on the neonatal renal blood flow at therapeutic doses (1-20 μg/kg/min) of dopamine.[7-12] Piglet studies of dobutamine showed no effect on renal blood flow despite increasing cardiac output.[10,13] Valverde *et al* found no significant difference in urine output or renal function in infants receiving dopamine or epinephrine except for different side effect profiles.[14]

Milrinone is a phosphodiesterase III inhibitor that can increase cardiac output and cause vasodilation through increased intracellular cyclic adenosine monophosphate (cAMP) concentration.[15,16] In a small study of adult dogs undergoing 15 min of hypoxia, bolus dosing of milrinone (25, 50 and 250 μg/kg) has been shown to increase renal blood flow in hypoxic dogs compared to placebo.[17] However, the effect of milrinone infusions at relevant dosing schedules in hypoxia-reoxygenation (H-R) was not determined. Despite the use of milrinone in a neonatal population for indications such as low cardiac

output post cardiac surgery and pulmonary hypertension, there currently is no data on the effect of milrinone on the neonatal renal vasculature. [18-20] The objective of this study was to determine the changes in renal perfusion: blood flow, vascular resistance and oxygen delivery with the administration of milrinone in an animal model of neonatal H-R.

METHODS

The methods have been previously described in Chapter 7. In addition, an incision through the left flank with minimal dissection, the left renal artery was identified and exposed. A 2-mm Transonic® flow probe (2 SB, Transonic Systems Inc, Ithica, NY) was placed around the renal artery to continuously monitor the blood flow to the left kidney throughout the experimental period. Left renal artery flow was recorded during hypoxia, reoxygenation and medication administration. Left renal artery flow index, renal vascular resistance index, and renal oxygen delivery index were calculated as shown in Appendix 4.

Results are expressed as mean±standard error of the mean. Hemodynamic variables were analyzed by 2-way ANOVA followed by 1- way repeated measures (RM) ANOVA for differences within groups over time and 1-way ANOVA for differences between groups at a given time point. Tukey or Dunnett's method were used for post hoc testing when appropriate. Analysis was performed using Sigma Stat (Version 2.0, Jandel Scientific, San Rafael, CA). Significance was defined as $p < 0.05$.

RESULTS

During stabilization, prior to the initiation of hypoxia, the left renal artery flow index (RAFI) was 8.7±0.7 mL/kg/min (5% of cardiac index). The left renal oxygen delivery ($RADO_2$) was 0.90±0.07 mL O_2 kg^{-1} min^{-1}. Left renal artery vascular resistance index (RAVRI) was 8.3±0.6 mmHg mL^{-1} kg^{-1} min^{-1}. Normoxic baseline cardiac index was 173±7 mL/kg/min with a mean arterial pressure (MAP) of 70±2 mmHg. There were no significant differences in the hemodynamic variables among the groups.

Hypoxia

During normocapneic alveolar hypoxia, RAFI was significantly reduced to 2.7±0.5 mL/kg/min (28±5% of normoxic baseline) as was $RADO_2$ to 0.11±0.02 mL O_2 kg^{-1} min^{-1} (12±2%). The RAVRI increased to 12.3±2.6 mmHg mL^{-1} kg^{-1} min^{-1} (154± 28%). The cardiac index was decreased to 71±4 mL/kg/min (41±1%) along with systemic hypotension (MAP of 28±2 mmHg; 41±2%). Hemodynamic variables at the end of hypoxia did not differ among hypoxic groups.

Reoxygenation

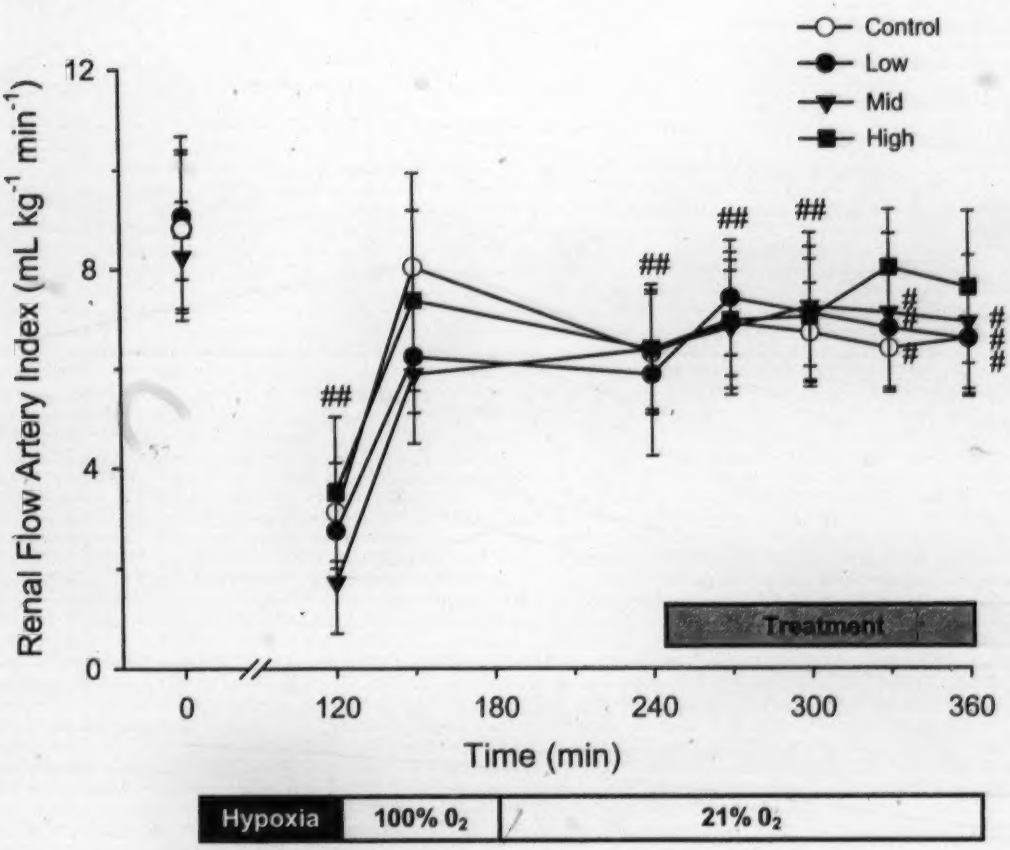
Upon reoxygenation with 100% oxygen, hemodynamics temporarily recovered to baseline values for 30 to 60 min. Over the next 60 min of reoxygenation with weaning of FiO_2 to 0.21, parameters deteriorated. The P_aO_2 was normalized (61±1 mmHg), with a pH of 7.38±0.01 at 2h of reoxygenation. The RAFI fell to 6.3±0.6 mL/kg/min (70±5%), $RADO_2$ was 0.62±0.07 mL O_2 kg^{-1} min^{-1} (68±3%) with a RAVRI was 7.5±0.9 mmHg mL^{-1} kg^{-1} min^{-1} (90±8%). Systemically, CI decreased to 75±3% and MAP to 67±3% of

normoxia baselines. No significant differences in hemodynamic variables were found between groups prior to medication delivery.

After Milrinone or Placebo Infusion

During the 2h infusion of milrinone there was no significant difference in RAFI between milrinone groups and hypoxic placebo controls (Figure 8-1). Although RAFI increased slightly after milrinone treatment, the increases were not significant among the groups (control:98±7%, low-dose:119±22%, mid-dose:111±9%, high-dose:135±26% of the respective pretreatment values). High dose milrinone was the only treatment group in which RAFI returned to values similar to normoxic baseline ($p>0.05$, RM ANOVA). The changes in RAVRI and $RADO_2$ mirrored that of RAFI after the infusions of milrinone were started but the changes were not significant (RAVRI: control:100±3%, low-dose:87±19%, mid-dose:90±6%, high-dose:89±10% pretreatment baseline; $RADO_2$: control:90±9%, low-dose:118±24%, mid-dose:105±10%, high-dose:130±31%) (Figures 8-2, 8-3).

FIGURE 8-1: RENAL ARTERY FLOW INDEX

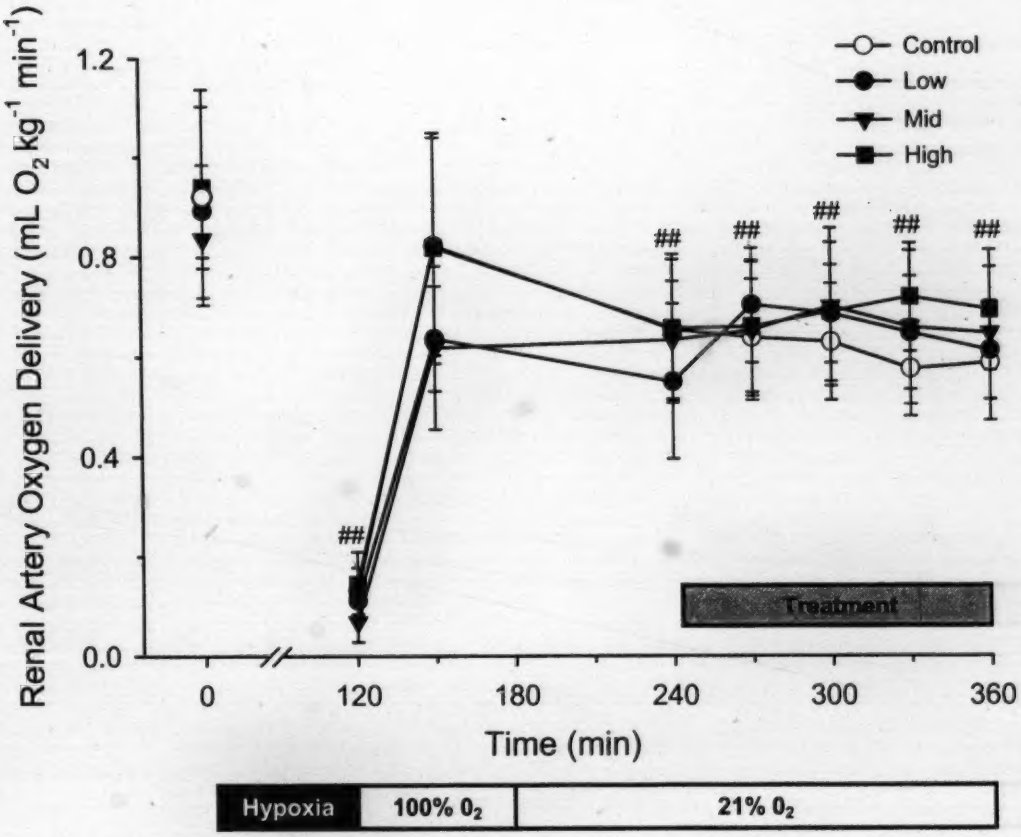


There was no significant difference among groups.

# $p<0.05$ vs. normoxic baseline (0 min)

$p<0.05$ all groups vs. normoxic baseline (0 min)

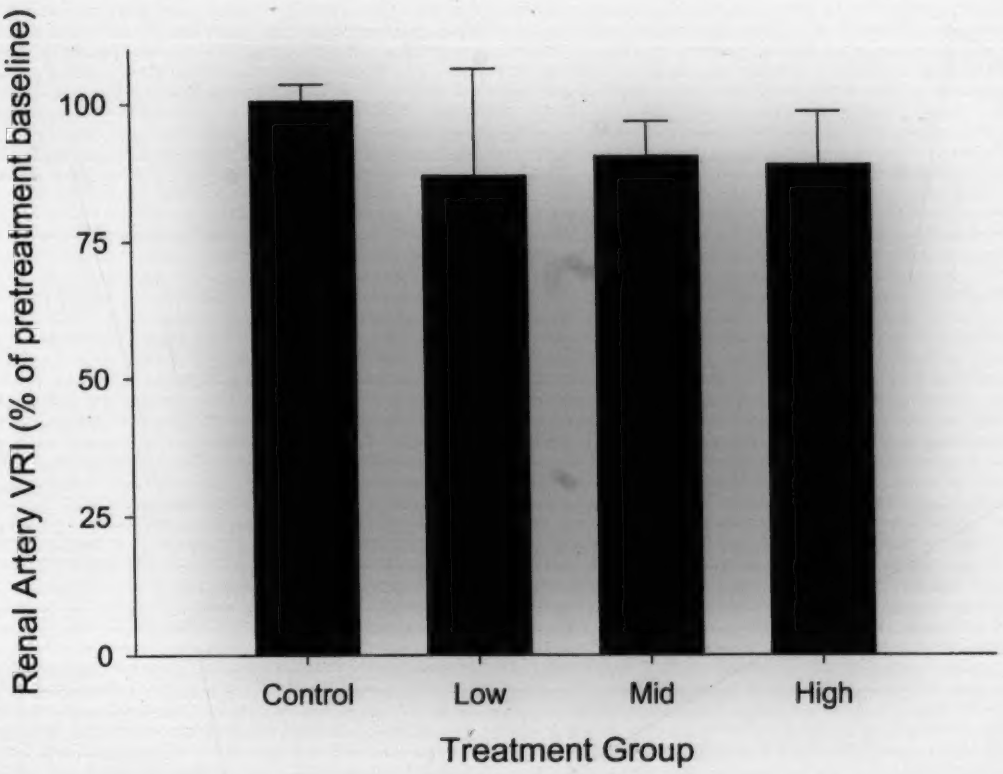
FIGURE 8-2: RENAL ARTERY OXYGEN DELIVERY



There was no significant difference among groups.

$p<0.05$ all groups vs. normoxic baseline (0 min)

FIGURE 8-3: RENAL ARTERY VASCULAR RESISTANCE INDEX (VRI)



There was no significant difference in renal artery VRI at the end of treatment (expressed as percentage of pretreatment baseline) among the groups.

DISCUSSION

Milrinone is a phosphodiesterase III inhibitor that increases the intracellular concentration of cAMP. Elevated cAMP concentration can increase cardiac index and cause vasodilation.

In our study, an infusion of milrinone did not significantly alter the recovery of renal blood flow or oxygen delivery during reoxygenation after 2h of severe hypoxia. The infusion of milrinone at 0.75 μg/kg/min may have a beneficial effect on the RAFI, restoring it to normoxic baseline values.

During normoxia, Fujishima's work in dogs has shown that milrinone can still increase cardiac index and decrease systemic vascular resistance without a significant effect on renal blood flow.[21] Our study also did not find a significant increase in renal blood flow in a state of normoxia after hypoxia.

This contradicts the findings of Setoyama *et al*, who used microspheres in adult dogs during 15 min of hypoxia in the presence or absence (control) of incremental doses of milrinone.[17] Setoyama *et al* showed an increase in renal blood with milrinone administration during hypoxia but did not investigate the effects of prolonged asphyxia, reoxygenation or clinically relevant dosing of milrinone. Microspheres only allowed for sporadic measurement of RAFI as opposed to continual changes in renal perfusion. The hypoxia response of renal blood flow in animal models can differ depending on the species as well as the duration and severity of hypoxia.[22]

This study showed that hypoxia markedly decreases renal blood flow and oxygen delivery with an increased vascular resistance. The difference in findings between Setoyama's and our own findings may indicate the milrinone's vasodilatory properties

may be effective in the renal vasculature during a state of increased vascular resistance as found in hypoxia but may not provide an additional benefit during prolonged reoxygenation.

A significant hemodynamic renal effect may be masked in the large variance in renal hemodynamic variables during times of H-R. This large variance was not found at normoxic baseline (<10% SEM), indicating that the variance was more likely due to physiological variation in the piglets' response to H-R and not the instrumentation.

In conclusion, in the decreased renal blood flow and oxygen delivery during reoxygenation after hypoxia in neonatal piglets, a short course of milrinone infusion (2h) does not augment recovery of renal hemodynamic variables over that of hypoxic placebo controls.

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CHAPTER 9

Conclusions and Future Directions

Neonatal asphyxia can result in shock and multiorgan dysfunction with increased pulmonary vascular resistance that occurs after resuscitation. The resulting low cardiac output state can lead to decreased blood flow and oxygenation to the peripheral organs.

Current treatments include the use of catecholamine infusions to provide inotropic support. However elevated doses of catecholamines may increase heart rate and cardiac function at the expense of increased oxygen consumption, decreasing peripheral blood flow and aggravating pulmonary hypertension. Milrinone is a specific phosphodiesterase III inhibitor that increases myocardial contractility and cardiac output with vasodilatory effects to alleviate increased vascular resistance. Further increase in myocardial oxygen consumption has not been observed.

This research has endeavored to determine if milrinone can improve the cardiac dysfunction, pulmonary hypertension, and regional hypoperfusion encountered clinically in neonates after severe asphyxia.

Our findings demonstrated that in a piglet model of neonatal hypoxia-reoxygenation, milrinone at clinically relevant doses can significantly increase cardiac output, stroke volume and systemic oxygen delivery with decreased systemic and pulmonary vascular resistance while maintaining the systemic blood pressure. Milrinone at a high-dose (0.75 μg/kg/min) can increase regional blood flow and oxygen delivery with decreased vascular resistance in the mesenteric and carotid circulations. We propose that the positive inotropic action of milrinone combined with its ability to ameliorate increased vascular resistance improves cardiac output and regional perfusion to the intestine and brain.

The increase in cardiac output, oxygen transport and blood flow to the peripheral organs was not associated with secondary cardiac tissue hypoxia or metabolic dysfunction as cardiac tissue did not demonstrate an increase in lactate or significant ischemic histologic changes after two hours of milrinone infusion.

While the intestinal tissue lactate and histology did not differ significantly among groups, there was a negative correlation between plasma milrinone and intestinal oxidized glutathione levels. Milrinone may play a beneficial role in decreasing oxidative stress and subsequently improving intestinal vascular resistance.

Given milrinone's ability to decrease systemic vascular resistance, there is a risk of lowering blood pressure in these asphyxiated neonates. This potential complication of the drug may occur especially if milrinone is infused into a system with inadequate circulatory volume. We did not observe significant hypotension associated with the milrinone infusion. Furthermore, there was no significant hemodynamic compromise during the loading phase of milrinone. Blood pressure was most likely maintained by the increased cardiac output. Literature indicates that cardiac output and alterations in peripheral vascular resistance have more influence on regional blood flow and oxygenation than blood pressure after neonatal asphyxia.

Milrinone is able to decrease pulmonary vascular resistance even at lower doses (0.25 μg/kg/min) of milrinone. Despite an increase in cardiac output, milrinone did not increase pulmonary arterial pressure, a finding associated with other catecholamines used at high doses in neonatal asphyxia.

Our findings are consistent with those from a small body of literature for the use of milrinone in neonates with low cardiac output and pulmonary hypertension. Similar

cardiopulmonary pathophysiology can also be found in asphyxiated infants. This research addresses a new use for milrinone in neonates after asphyxia. Given the current use of milrinone in small, specific neonatal subsets of disease, this research also addresses the paucity of literature and effectiveness of milrinone on regional perfusion and tissue energetics that should be available to help clinicians make appropriate treatment decisions in neonates.

In conclusion, this research has documented the effects of milrinone on systemic and regional hemodynamics when used to treat shock in hypoxia- reoxygenation in newborn pigs. Clinical studies are needed to confirm our observations in humans neonates affected by the sequelae of asphyxia.

Future Directions

Although milrinone was effective in a piglet model of neonatal hypoxia-reoxygenation, further studies are required before applying these results to human asphyxiated neonates.

Tissues and plasma collected during this research can be further analyzed with respect to the possible effects of milrinone on cardiac and organ energetics (such as tissue levels of cAMP, ATP, ADP and AMP kinase) to ensure that increased cardiac work and organ blood flow is beneficial at the cellular level.

As this study examined the systemic and regional hemodynamics for a short duration in acutely instrumented animals, chronic animal protocols should be done to examine the effects of prolonged infusions of milrinone. Clinically relevant laboratory tests of organ function (plasma levels of creatinine and troponins, and clotting times)

could done in conjunction with less invasive measures of cardiac function and regional perfusion that are translatable to the bedside (echocardiography, near infrared spectroscopy, Doppler ultrasound of regional vessels). Chronic studies may also allow the manifestation of sequalae in the clinical, chemical, and histopathological realms. In concert with these studies, an attempt to determine the contributions of cardiac output, regional blood flows, or blood pressure to animal and organ outcomes should be made.

A comparison of milrinone to other inotropes currently used in neonatal intensive care units to treat the sequelae of neonatal asphyxia should be performed in models as a step towards future clinical trials.

Examination of neonates currently receiving milrinone should be undertaken to try and correlate milrinone levels, cardiac function, pulmonary pressure, organ perfusion, clinically relevant side effects (thrombocytopenia, dysrhythmias), and developmental outcome. Participation in multicentre randomized trials may provide more information on the neonate's ability to tolerate and benefit from milrinone at clinically relevant doses.

To mimic some of the more urgent and severe forms of perinatal asphyxia, the questions could be addressed using models of acute fetal events including placental abruption or cord prolapse with subsequent resuscitation and inotrope administration. Variations in the oxygen concentration during resuscitation and allowing the carbon dioxide concentration to rise during asphyxia and resuscitation may provide further avenues of research.

Although this research has provided some answers with respect to milrinone use in a model of neonatal asphyxia-reoxygenation, many questions remain to be answered in the hopes of providing optimal intensive care for sick neonates.

APPENDIX 1

Surgical Instrumentation

Figure A-1 : Surgical Team: Dr. Joynt, C. Cote, and J. Li during instrumentation

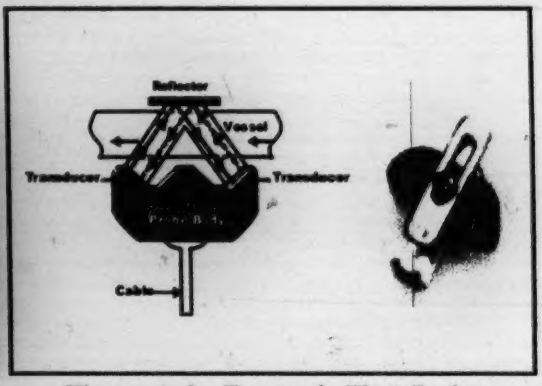


Figure A-2: Transonic Flow Probe

Figure A-3: Right femoral arterial and venous catheterization

Figure A-4 : Pulmonary artery flow probe, pressure catheter and PDA clip

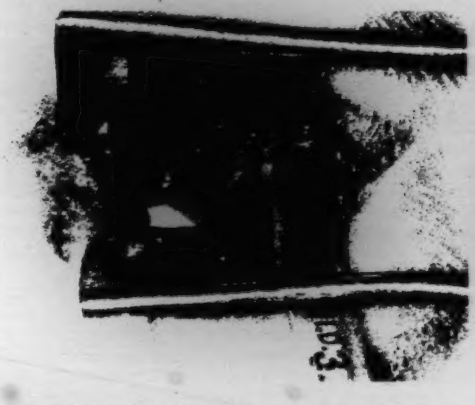
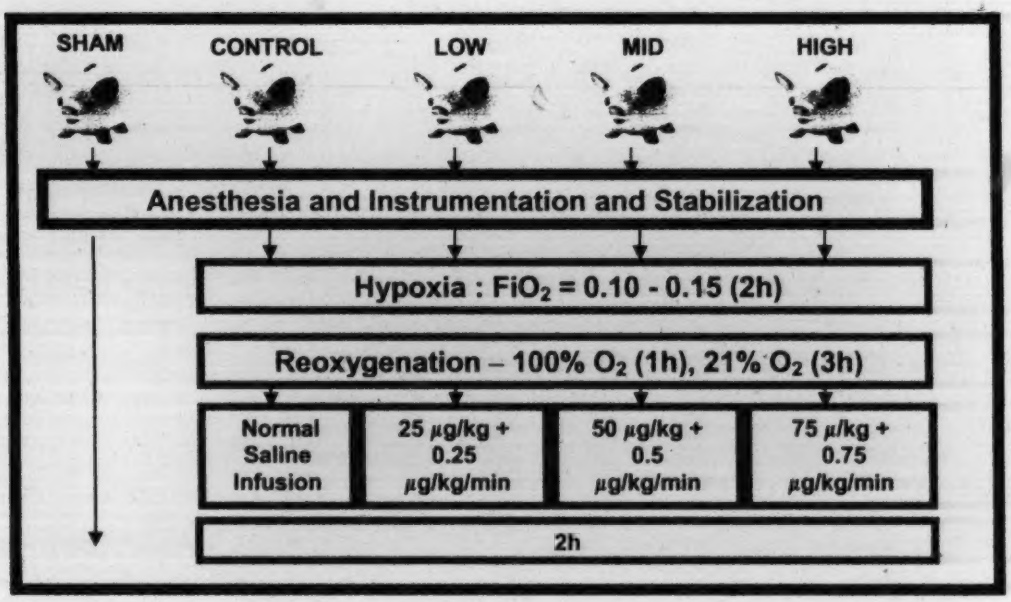


Figure A-5: Right common carotid artery flow probe and tracheostomy

Figure A-6: Superior mesenteric artery probe, isolated renal artery

APPENDIX 2

Experimental Protocol Model of Milrinone in Neonatal Hypoxia-Reoxygenation



APPENDIX 3

Protocol for Hypoxia-Reoxygenation Piglet Model

Time (min)	Action / Comment	Record Parameters*	Blood for Lactate	Co-oximetry†
Prior to Study	Anaesthesia of piglets Surgical instrumentation			
Stabilization 20 min	Post operative recovery FiO_2 of 0.21			
0	Baseline recording /bloodwork Initiate hypoxia FiO_2 0.10-0.15	X	X	X
30		X		
60		X	X	X
90		X		
120	2 hr hypoxia, Record/ blood Start reoxygenation FiO_2 1.0	X	X	X
130	10 min of reoxy/ reperfusion FiO_2 100%	X	X	X
150	30 min of reoxy FiO_2 1.0	X	X	X
180	60 min of reoxy FiO_2 1.0; Recording/blood Turn down FiO_2 to 0.21	X	X	X
240	2h of reoxy – 60 min at 21% Record/ bloodwork Start medication/ placebo 15 min bolus then infusion rate	X	X	X
270	150 min reoxy, 30 min infusion	X		X
300	3h reoxy, 1h infusion	X	X	X
330	210 min reoxy, 90 min infusion	X		X
360	4h reoxy, 2h infusion	X	X	X
Study done	Euthanasia of piglet Harvest tissue samples			

* Parameters recorded : Fraction of inspired oxygen (FiO_2), heart rate, oxygen saturations, mean arterial pressure, pulmonary arterial pressure, central venous pressure, pulmonary artery flow, regional artery flow (carotid, superior mesenteric, renal), temperature

† Mixed venous (pulmonary artery) and arterial (femoral artery) samples.

APPENDIX 4

Hemodynamic Variable Calculations

Hemodynamic Variable	Calculation
Cardiac index (CI)	Cardiac output ÷ weight (kg)
Stroke volume index	CI ÷ heart rate
Systemic vascular resistance (SVRI)	(MAP – CVP) ÷ CI
Estimated pulmonary vascular resistance (PVRI)	PAP ÷ CI
Arterial oxygen content (C_aO_2)	(1.39 x Hgb[v] x S_aO_2) + (0.003 x P_aO_2)
Systemic oxygen delivery	C_aO_2 x CI
Systemic oxygen consumption	CI x [1.39 x Hgb x (S_aO_2 – S_vO_2[vi]) + (0.003 x P_aO_2)]
Systemic oxygen extraction	[(S_aO_2 - S_vO_2) ÷ S_aO_2] x 100
Carotid artery flow index (CAFI)	Carotid flow ÷ weight (kg)
Superior mesenteric artery flow index (SMAFI)	SMA flow ÷ weight (kg)
Renal flow index (RAFI)	Renal flow ÷ weight (kg)
Carotid vascular resistance index	(MAP-CVP) ÷ CAFI
Estimated SMA vascular resistance index	MAP ÷ SMAFI
Renal vascular resistance index	(MAP-CVP) ÷ RAFI
Carotid oxygen delivery index	C_aO_2 x CAFI
SMA oxygen delivery index	C_aO_2 x SMAFI
Renal oxygen delivery index	C_aO_2 x RAFI

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[v] Hgb = Hemoglobin concentration (g/dL)
[vi] S_vO_2 = Venous oxygen saturation

APPENDIX 5

Plasma Lactate Protocol

Formula to Calculate Lactate Concentration[vii]

$$C = \frac{V * MW}{\varepsilon * d * v * 1000} * \Delta A \text{ (g/L)}$$

C = concentration of lactate
V = final volume (mL)
v = sample volume (mL)
MW = molecular weight of substance assayed (lactate = 90.1 g/mol)
d = light path (1 cm)
ε = extinction coefficient of NADH at 340 nm (6.3 $L*mol^{-1}*cm^{-1}$)

Plasma Lactate Assay Reaction

L-lactate is oxidized to pyruvate by nicotinamide- adenine dinucleotide (NAD) when exposed to L-lactate dehydrogenase (L-LDH).

$$\text{Reaction 1 : L- Lactate} + NAD^+ \xleftrightarrow{\text{L-LDH}} \text{pyruvate} + NADH^+ + H^+$$

The equilibrium of this reaction favors L-lactate. The equilibrium can be shifted to favor pyruvate and NADH by using pyruvate in a subsequent reaction induced by the enzyme glutamate- pyruvate transaminase (GPT) in the presence on L-glutamate.

$$\text{Reaction 2: Pyruvate + L-glutamate} \xleftrightarrow{\text{GPT}} \text{L-alanine + 2 cyclo oxoglutarate}$$

The NADH produced in reaction 1 is stoichiometric to the amount of L-lactic acid. The concentration of NADH can be determined via light absorbance at 340 nm.

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vii L-lactate UV method enzymatic bioanalysis. Boehringer Mannheim/ R- Biopharm. Catalogue Number 10 139 084 035. pg 1-3.

APPENDIX 6

Tissue Lactate Protocol

SOLUTIONS TO MAKE UP PRIOR TO PROTOCOL (make fresh each day)

1) 6% PCA / 0.5 mM EGTA

- Take 2.5 cc of 60% perchloric acid stock and add to 22.5 cc ddH_2O (to make a total of 25cc) and add 4.75 mg of EGTA

2) 5 M K_2CO_3

- add 3.46 g K_2CO_3 to 4.3 cc of ddH_2O

PROTOCOL

1) Take 50 mg of crushed frozen tissue (know exact weight) preweighed in flat bottom centrifuge tube

2) Add 500 µL of 6% PCA / 0.5 mM EGTA (per 50 mg-calculate volume equivalent for each weight) to crushed tissue in centrifuge tube and homogenize

3) Sit tube on ice for 10 min

4) Centrifuge at 11,000 rpm for 2 min in microcentrifuge

5) While centrifuging, weigh another centrifuge vial

6) Transfer supernatant to the preweighed centrifuge tube and weigh again to determine weight (i.e. volume) of supernatant

7) Add 5 M K_2CO_3 in ratio of 50 µL K_2CO_3 : 500 µL supernatant . You will have to calculate the volume of K_2CO_3 needed from the volume of supernatant you measured in step 6. ADD DROP by DROP as can bubble over

8) Sit tube on ice for 30 min. A precipitate should form.

9) Microcentrifuge at 11,000 rpm for 2 min, while tube is spinning weigh another vial.

10) Transfer supernatant to preweighed vial. Determine final supernatant volume. If not using now, store supernatant in -80°C fridge (can also use for protein determination)

11) Use 50 µL of supernatant for the plasma lactate protocol

13) Prepare blank for plate reader- 45.5 µL 6% PCA / 0.5 mM EGTA + 4.5 µL K_2CO_3

14) With plate reading, increase is $\approx$ 0.05 every 5 min and takes about 90 min to finish